Development of bacterial systems for the production of cellulase and bioethanol

A thesis presented to

The Faculty of Graduate Studies

of

Lakehead Univeristy

by

MIRANDA MAKI

In partial fullfillment of the requirements
for the degree of
Doctor of Philosophy in Biotechnology

August 30th, 2012

Abstract

The wide varieties of extant bacterial species are often resistant to various environmental stresses. This demonstrates their frequent ability to adapt to and thrive in challenging environments. One such adaptation in wood-degrading species that may be exploited to produce a product of high value to humans is a more efficient cellulase activity, which may help to overcome current challenges in biofuel production. In this study, 18 efficient cellulase-producing bacteria were isolated from organic fertilizers and paper mill sludges and characterized for consideration in large scale biorefining. All cellulase positive isolates were further characterized to identify those with the greatest cellulase activities for potential industrial application. Six of these isolates produced greater cellulase activity on soluble cellulose in 48 h than the positive control (*Cellulomonas xylanilytica*). Phylogenetic analysis of a portion of the 16S rDNA gene revealed genera belonging to two major phyla of Gram positive bacteria: Firmicutes and Actinobacteria. Additionally, isolates E2 and E4 (*Paenibacillus* species) displayed qualitative cellulase activities towards filter paper under limited oxygen condition. When total cellulase activities of E2 and E4 were examined, it was shown that 1% (w/v) carboxymethyl cellulose (CMC) could induce total cellulase activities of 1652 ± 62 and 1457 ± 31 nM of glucose equivalents that were 8- and 5.6-fold greater than total cellulase activities induced by filter paper for E2 and E4, respectively. The genus *Paenibacillus* includes many highly-expressing cellulase producing strains, and E2 and E4 represent excellent candidates for further cellulase activity analysis and characterization.

Cellulose hydrolysis is only one of the rate-limiting steps in the industrial production of biofuels which can be improved by isolation and characterization of novel enzymes. In addition, pretreatment of lignocellulosic biomass is a costly hurdle which can be improved by the application of bacteria capable of producing a greater variety of enzymes. The potential use of lignocellulosic biomass for biofuel production has been hampered due to the complexity of its composition and the lack of microorganisms capable of modifying or decomposing the different components. Thus, CMC-containing agar was used to isolate and characterize 20 cellulase-producing bacterial strains from peat and municipal wastes that belonged to four major phyla:

Firmicutes, Actinobacteria, Proteobacteria and Bacteriodetes. Seven of the cellulase positive isolates also exhibited filter paper activities, while 13 exhibited activities towards xylan. Moreover, 10 of the isolates were capable of surviving 21 days incubation with 1% black liquor. Five strains increased the absorbance of black liquor by greater than 10-fold. Similarly, these five strains could also increase the absorbance of lignin at 280 nm when grown with 0.1% pure lignin. Additionally, although FTIR analysis of 1% barley straw treated for 21 days with these 5 strains showed a preference for consumption of hemicelluloses over lignin, a change in lignin was observed. Two isolates, 55S5 and AS1, a *Bacillus* sp. and *Pseudomonas* sp., respectively, have the highest lignocellulase activity, that is activities towards cellulose, hemicellulose and lignin, and possess the greatest potential for industrial use because of their concomitantly high cellulase activities, including filter paper activity and in addition, xylanase activity.

The anaerobic, thermophilic and ethanogenic bacterium *Clostridium thermocellum* has great potential for use in consolidated bioprocessing for a more cost effective production of biofuels. However, its application is still hindered by such obstacles as end-product inhibition, i.e. feedback inhibition to cellulase activity by cellobiose. To increase cellulase activity and ethanol production, the copy number of β-glucosidase A (*bgl*A*)* in *C. thermocellum* 27405 was increased using shuttle vector pIBglA to lower the end-product inhibition of cellulase. Using a modified electrotransformation protocol, *C. thermocellum* transformant (+MC*bgl*A) harbouring pIBglA was successfully produced. The β-glucosidase activity of +MC*bgl*A was 2.3- and 1.6-fold greater than wild-type (WT) during late log and stationary phases of growth, respectively. Similarly, total cellulase activity of +MC*bgl*A was shown to be 1.7-, 2.3- and 1.6-fold greater than WT during, log, late log and stationary phases of growth. However, there was no significant correlation found between increased cellulase production and increased ethanol titres for +MC*bgl*A compared to the WT, perhaps due to the accumulation of toxic end-products (i.e. ethanol). We successfully increased total cellulase activity by increased expression of *bgl*A and thereby increased the productivity of *C. thermocellum* during the hydrolysis stage in consolidated bioprocessing. Our work also provides insight into the complex metabolism of *C. thermocellum* for future further improvement of this strain.

The co-culture of *Clostridium thermocellum* and *Thermoanaerobacterium saccharolyticum* has great potential in the production of biofuels because it will consolidate the hydrolysis and fermentation steps and potentially increase bioethanol titres. However, there is little knowledge of the industrial application of this kind of co-culture such as substrate conditions and the number of generations for stable co-culture in addition to the effect of ethanol titres. The goal of this study was to develop a stable co-culture of *C. thermocellum* 27405 and *T. saccharolyticum* 31097 which can produce greater ethanol titres than mono-cultures in batch fermentation. Comparison of *C. thermocellum* and *T. saccharolyticum* growth in reducing sugar (1% (w/v) cellobiose and 0.5% (w/v) xylose) and polysaccharide (1% (w/v) Avicel and 0.5% (w/v) cellobiose) media, showed that *T. saccharolyticum* could grow 2-fold faster in reducing sugar medium compared to *C. thermocellum*, while *C. thermocellum* grew to 2.3-fold greater turbidity in polysaccharide medium in mono-cultures. Subsequent co-culture batch cultures revealed that both strains could only co-exist for complete cell culture in reducing sugar medium, as confirmed by biomarker genes (*bgl*A and *xyl*B, respectively) detected by PCR, while in the subsequent subcultures only *T. saccharolyticum* was detected. In polysaccharide medium, both strains were detected continuously for 4 generations in batch culture trials, using the same biomarker genes. After the fourth continuous subculture, the co-culture required re-establishing or further media optimization due to growth inhibition of strains. Additionally, the ethanol titres also increased by 2.01-fold in the first and second subcultures compared to the mono-cultures. However, third and fourth subcultures did not have significantly different ethanol titres. Nonetheless, *C. thermocellum* and *T. saccharolyticum* co-culture has potential application if added during the hydrolysis stage of complex polysaccharides but not if added to simple sugars such as short poly- and oligo-saccharides produced during the fermentation stage.

All of the work presented here in this thesis, focuses on the potential exploitation of bacteria to improve the economic feasibility of biofuels from stages of pretreatment, to hydolysis and fermentation. Due to the large variety and extreme environmental resistance, as well as genetic advances in prokaryotic

systems the potential to improve exisiting bacterial systems or isolate new strains for industrial application is immense.

List of Abbreviations

ABTS – 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid)

BMCC – Bacterial microcrystalline cellulose

BTP – 2-(2''-benzothiazolyl)-phenyl

CBD – Carbohydrate binding domain

CBH – Cellobiohydrolases

CBM – Carbohydrate binding module

CBP – Consolidated bioprocessing

CbpA – Scaffolding protein of *Cellumonas cellulovorans*

CMC – Carboxymethyl cellulose

CMCase – Carboxymethyl cellulase

CFLSM – Confocal scanning laser microscopy

DAM – DNA adenine methylase

DOGS – Degenerate oligonucleotide gene shuffling

DP – degree of polymerization

DS – degree of substitution

DTAF – 5-(4,6-dichlorotriazinyl) aminofluorescein

EG – Endoglucanases

EngB – Endonuclease B

epPCR – error-prone polymerize chain reaction

FPA – Filter paper activity

FP – Filter paper

FPU – Filter paper unit

GH – Glycoside hydrolases

GOD – Glucose oxidase

HBT – Hydroxybenzotriazole

HPLC – High performance liquid chromatography

Inp – Ice nucleation protein

IUPAC – Internation Union of Pure and Applied Chemistry

LB – Luria Bertani

MU-C – MU-β-D-cellobioside

NaAc – Sodium acetate

PBS – Potassium phosphate buffer

pNPG – 4-nitrophenol β-D-glucuronide

PSC – Phosphoric acid-swollen cellulose

RSE – Reducing sugar equivalents

SFC – Saccharification-co-fermentation

SLH – Surface layer homology

QCM – Quartz crystal microbalance

WT – wild-type

Xyl – Xylanase

Acknowledgments

First of all, I am deeply grateful to my superviors Dr. Wensheng Qin and Dr. Kam Leung for all of their guidance, encouragement, and financial support, during my Ph.D. study. I would also like to extend my sincere thanks to the members of my supervisory committee Dr. David Law and external examiner Dr. Hung Lee, for sharing their valuable time by lending their knowledge and support to my thesis.

Moreover, I wish to express sincere gratitude to the hard working honors thesis students who worked with me throughout my thesis: Lachlan Armstrong, Amna Idrees and Michael Broere. I am also very grateful to Susanne Walford for collecting samples and assisting in project analyses; and also Bruce Rosa for his expertise in computer software, data analyses and advice. This work would not be complete without their help. Also, a very special thank you to Dr. Schraft and Dr. Leung for sharing their lab equipment and bench space while our lab was under construction.

I would also like to especially thank the support of all the members of Dr. Qin‟s laboratory over the period of my Ph.D. for sharing their knowledge with me and eagerly lending a hand in times of need. In addition, I further extend thanks to speedy and pleasant technical support from Greg Kepka, technician of Lakehead Univerisity‟s Instrumentation Lab and Biorefining Institute.

This work was further made possible by the donation of plasmid pIKM1 from Dr. Lee Lynd of Dartmouth University. Also, special thanks for the financial support from NSERC and OGS throughout my doctoral study.

Finally, it is with much gratitude and sincerity I thank my mother, family and friends for love and patience, and for encouraging me throughout the many adventures of my Ph. D.

Table of Contents

List of Tables

List of Figures

Chapter 5: Development of a stable *Clostridium thermocellum* and *Thermoanaerobacterium* co-culture for industrial production of bioethanol

Chapter 1: Literature Review

1.1 The prospects of cellulase-producing bacteria for the bioconversion of lignocellulosic biomass

Authors: Miranda Maki, Kam Tin Leung, Wensheng Qin.

Abstract:

Lignocellulosic biomass is a renewable and abundant resource with great potential for bioconversion to value-added bioproducts. However, the biorefining process remains economically unfeasible due to a lack of biocatalysts that can operate industrial conditions such as at high temperatures, under reduced oxygen/stirring, and in acidic or basic pH. The extreme environmental resistance of bacteria permits their screening for novel thermo-, hypoxia- and pH-tolerant cellulases that may help overcome these challenges. Rapid, efficient cellulase screening techniques, using cellulase assays and metagenomic libraries, are a must. Rare cellulases with activities on soluble and crystalline cellulose have been isolated from strains of *Paenibacillus* and *Bacillus* and shown to have high thermostability and/or activity over a wide pH spectrum, while novel cellulases from strains like *Cellulomonas flavigena* and *Terendinibacter turnerae* are multifunctional with a broader substrate utilization. These enzymes offer a framework for enhancement of cellulases including increasing specific activity and thermostability and/or reducing end-product inhibition. In addition, anaerobic bacteria like *Clostridia* sp. offer potential because they may produce multienzyme complexes called cellulosomes. Cellulosomes provide synergy and close proximity of enzymes to substrate, increasing activity towards crystalline cellulose. This has lead to the construction of designer cellulosomes enhanced for specific substrate activity. For example, cellulosome-producing *Clostridium thermocellum* has a high ability to ferment sugars to ethanol and is amenable to co-culture and is promising for biofuel production thanks to recent advances in genetic engineering. The exploitation of bacteria in the search for improved enzymes or strategies provides a means to upgrade feasibility for lignocellulosic biomass conversion, ultimately providing means to a „greener" technology.

Introduction:

The combustion of petroleum-based fossil fuels has become a concern with respect to global climate change due to accelerated carbon emissions [1]. Burning of fossil fuels has also created a concern for unstable and uncertain petroleum sources, as well as, the rising cost of fuels [2]. These concerns have shifted global efforts to utilize renewable resources for the production of a „greener‟ energy replacement which can also meet the high energy demand of the world. The Canadian renewable fuel standard has been raised so that fuel will contain 5% ethanol by 2010 [3]; the US Environmental Protection Agency raised their renewable fuel standard to 10.21% ethanol mixed fuels by 2009 [4]; while, the current mandate for mixing ethanol in fuel for Brazil is 25% (set in 2007) [5].

Currently, the US and Brazil are leaders in the production of starch/sugar-based fuels from corn and sugarcane crops, respectively. This is the production of first generation fuel from food-crop sugars using conventional technologies; however, starch raw materials will not be sufficient enough to meet increasing demand and are a controversial resource for bioconversion [6]. Also the reduction in greenhouse gases is low for starch-based ethanol and thus, second generation fuels based on non-edible crops (lignocellulosic biomass), is gaining immense global and scientific attention.

Lignocellulosic biomass, („plant biomass‟), is a great potential resource for the production of biofuels because it is largely abundant, inexpensive and production of such resources is environmentally sound. Agricultural residues are a great source of lignocellulosic biomass which is renewable, chiefly unexploited, and inexpensive. Such resources include: leaves, stems, and stalks from sources such as corn fibre, corn stover, sugarcane bagasse, rice hulls, woody crops, and forest residues. Also, there are multiple sources of lignocellulosic waste from industrial and agricultural processes, e.g., citrus peel waste, sawdust, paper pulp, industrial waste, municipal solid waste, and paper mill sludge. In addition, dedicated energy crops for biofuels could include perennial grasses such as Switchgrass and other forage feedstocks such as *Miscanthus*, Bermuda grass, Elephant grass, etc [6]. Approximately 70% of plant biomass is

locked up in 5- and 6-carbon sugars. These sugars are found in lignocellulosic biomass, which is comprised of mainly cellulose (a homologous polymer comprised of long chains of glucose); less so, hemicelluloses (heterologous polymer of 5- and 6-carbon sugars); and least of all lignin (a complex aromatic polymer). The major component cellulose, is a homopolysaccharide comprised of glucose units, linked by β-(1→4) glycosidic bonds. Cellobiose is a repeating unit of cellulose which is comprised of two glucose monomoners linked via β-(1→4) glycosidic bonds and can ultimately be converted into glucose. Hemicellulose is a heterogeneous polymer, which varies in composition from plant to plant and within different parts of the same plant. It is made up of mainly pentoses (D-xylose, L-arabinose), hexoses (D-mannose, D-glucose, D-galactose) and sugar acids (uronic and acetic acid). In hardwoods hemicellulose contains mainly xylans, while in softwood mainly glucomannans are present. Hydrolysis of hemicelluloses requires various types of enzymes. Briefly, xylan degradation requires endo-1-4,-β-xylanase, β-xylosidase, α-glucuronidase, α-L-arabinofuranosidase, as well as acetylxylan esterases. In glucomannan degradation β-mannanase and β-mannosidase are required to cleave the polymer backbone.

There are several advantages for the production of biosolvent fuels such as bioethanol: 1) produced from a variety of raw materials; 2) it is non-toxic to the environment; and 3) easily introduced into the existing infrastructure [7]. However, the path to sustainable and economically feasible biofuels is hampered. There are a few major bottlenecks with the current production of biofuels; one being, there is a lack of biocatalysts that can work efficiently and inexpensively at high temperatures and/or low pH conditions used in the bioconversion of lignocellulosic material to bioethanol. Moreover, there is a great need for cost-effective fermentation of sugars derived from cellulose and hemicellulose. Currently, industrial bioconversions of lignocellulose requires the application of high temperature and acidic or sometimes basic conditions to break down lignin, decrease crystallinity, increase pore volume and solubilise cellulose and hemicellulose to allow enzymatic hydrolysis of target polysaccharides [8]. This process is both expensive and inefficient. It is therefore important that enzymes be stable and active at high temperatures and/or low or high pH conditions.

Additionally, industrial bioconversion of lignocelluloses to ethanol occurs in multiple steps, where hydrolytic enzymes are added after pre-treatment of the lignocelluloses (saccharification) and then in an additional step, microorganisms capable of fermentation are added to the resulting monosaccharides generated during hydrolysis to ferment sugars to bioethanol. The multiple steps of the current biorefining process makes it both time-consuming and costly. By combining saccharification with fermentation in a process referred to as consolidated bioprocessing (CBP) or saccharification-co-fermentation (SCF), using a whole-cell(s) based approach, costs of fermentation and hydrolysis could be reduced [9,10]. Some additional rate-limiting steps in the bioconversion of lignocelluloses are the crystalline recalcitrance of cellulose and the limited number of cellulases. That is, all cellulolytic strains identified express low amounts in one or more type of glycoside hydrolases (GH) required for efficient cellulose hydrolysis (endo-/exo-glucanases, β-glucosidases). In attempts to improve the feasibility of the bioconversion of lignocellulose to biofuel, enzymes must have high adsorption capabilities, high catalytic efficiencies, high thermal stability and low end-product inhibition.

However, in addition to technical challenges there remain many ethical issues regarding the development of biofuels which can be addressed through revised policies and regulation. These issues include namely: negative effects towards food security; the rights of farmers and landholders in developing countries; and the environment. Furthermore, several claims have contested the ability of biofuels to significantly reduce greenhouse gase emissions [7].

Both fungi and bacteria have been heavily exploited for their abilities to produce a wide variety of cellulases and hemicellulases. Most emphasis has been placed on the use of fungi because of their capability to produce copious amounts of cellulases and hemicellulases which are secreted to the medium for easy extraction and purification. In addition, the enzymes are often less complex than bacterial glycoside hydrolases and can therefore be more readily cloned and produced via recombinant DNA methods in a rapidly growing bacterial host such as *E. coli*. However, the isolation and characterization of novel glycoside hydrolases from Eubacteria are now becoming widely exploited. There are several

7

reasons for these shifts, for one, bacteria often have a higher growth rate than fungi allowing for higher recombinant production of enzymes. Secondly, bacterial glycoside hydrolases are often more complex and are often expressed in multi-enzyme complexes providing increased function and synergy. Most importantly, bacteria inhabit a wide variety of environmental and industrial niches, which enrich for cellulolytic strains that are extremely resistant to environmental stresses. These include strains that are thermophilic or psychrophilic, alkaliphilic or acidiophilic, and, strains that are halophilic. Not only can these strains survive the harsh conditions found in the bioconversion process, but they often produce enzymes that are stable under extreme conditions which may be present in the bioconversion process and this may increase rates of enzymatic hydrolysis, fermentation, and, product recovery. Researchers are now focusing on utilizing, and improving these enzymes for use in the biofuel and bioproduct industries.

This review will focus on aspects rarely covered by other reviews, such as bacterial screening techniques, and new bacterial cellulases by comparing different cellulase-producing bacteria. Moreover, it will examine how these new cellulases can help overcome some of the major bottlenecks in the biofuel industry. In addition, this review will address how some novel bacterial strategies in biotechnology can advance the growing field of biorefining.

Bacterial cellulases:

Cellulases are comprised of independently folding, structurally and functionally discrete units called domains or modules, making cellulases modular [11]. A typical free cellulase is composed of a carbohydrate binding domain (CBD) at the C-terminal joined by a short poly-linker region to the catalytic domain at the N-terminal. There are only two modes of action for the hydrolysis of cellulose by cellulases, either inversion or retention of the configuration of the anomeric carbon. At least two amino acids with carboxyl groups located within the active site catalyze the reaction by acid-base catalysis. The commonly described mode of action for cellulases on polymers is either exo- or endo-cleavage, and all cellulases target the specific cleavage of β-1,4-glycosidic bonds [12]. Using this classification system,

8

cellobiohydrolases (exoglucanases) were classified as exo-acting based on the assumption that they all cleave β-1,4-glycosidic bonds from chain ends. As well, those enzymes truly exo-acting often have a tunnel-shaped closed active site which retains a single glucan chain and prevents it from re-adhering to the cellulose crystal [13-15]. While endoglucanases on the other hand, are often classified as endo-acting cellulases because they are thought to cleave β-1,4-glycosidic bonds internally only and appear to have cleft-shaped open active sites. Endoglucanase are active on amorphous regions of cellulose and thus their activity can be assayed using soluble cellulose substrates; i.e., the carboxymethylcellulase assay (CMCase). However, there is now supporting evidence that some cellulases display both modes of action, endo- and exo- [16]. Thus classification has changed; cellobiohydrolases (exoglucanases) are described as active on the crystalline regions of cellulose; whereas, endoglucanases are typically active on the more soluble amorphous region of the cellulose crystal. There is a high degree of synergy seen between cellobiohydrolases (exoglucanases) and endoglucanases, and it is this synergy that is required for the efficient hydrolysis of cellulose crystals.

CBD is the most common accessory module of cellulases and there are 54 distinct families [17]. The major function of CBDs is to deliver its resident catalytic domain to crystalline cellulose. Binding brings the catalytic domain into close contact with the crystalline cellulose for efficient hydrolysis. Binding of the cellulase via CBD is extremely stable, yet still allows the enzymes to diffuse laterally across the surface of the substrate and in some cases CBD has also been shown to catalyze the disruption of noncovalent interactions between cellulose chains of crystalline cellulose. Some other CBDs bind preferentially to noncrystalline cellulose [18-20].

Interestingly, the family 9 cellulase of aerobic *Thermomonospora fusca* has a family IIIc CBD with a different function that gives family 9 cellulases their distinctive theme [21]. This unique CBD does not bind crystalline cellulose but instead directly assists the catalytic function of the cellulase by binding a single cellulose chain and ultimately feeding this chain into the active site of the enzyme [22,23]. This

contributes to the overall processivity of the family 9 cellulase, that is, the sequential cleavage of the cellulose chain. Additionally, a second type of CBD must be associated with this cellulase to bind it to the crystalline cellulose. Moreover, the family 9 cellulase of *T. fusca* provides strong evidence for enzymes that can exhibit both endoglucanase and exoglucanase activities accentuating the equivocacy of these terms.

The products of exoglucanases and cellobiohydrolases, that are cellobiose and cellodextrans, respectively, are inhibitory to their activity. Thus, efficient cellulose hydrolysis requires the presence of β-glucosidases to cleave the final glycosidic bonds producing glucose. Typically cellobiose and cellodextrins are taken up by the bacteria and internally cleaved via cellodextrin phosphorylases or cellobiose phosphorylases to create glucose monophosphate, which is energetically favoured. Some bacteria also produce intra- or extra-cellular β-glucosidases to cleave cellobiose and cellodextrins and produce glucose to be taken up by or assimilated by the cell [17].

Screening and isolation of cellulase-producing bacteria:

Over the years, culturable, cellulase-producing bacteria have been isolated from a wide variety of sources such as composting heaps, decaying plant material from forestry or agricultural waste, the feces of ruminants such as cows, soil and organic matter, and extreme environments like hot-springs, to name a few [24]. Screening for cellulase production can be done by enrichment growth on microcrystalline cellulose as a sole source of carbon, followed by the extraction of 16S rDNA/RNA to determine the microbial community structure of the environment and analyze whether bacterial genera containing cellulase-producing species are present. Strains with cellulase potential can be isolated by subculturing from the enrichment culture on cellulose as a sole carbon source. This method was used to identify cellulase-producing bacteria in the deep subsurface of the Homstake gold mine, Lead, South Dakota, USA [25].

Moreover, efficient plate-screening methods are a prerequisite to finding cellulase-producing bacteria. Screening for bacterial cellulase activity in microbial isolates is typically performed on carboxymethylcellulose (CMC) containing plates [26]. This method can be time consuming and zones of hydrolysis are not easily discernable. Recently, Kasana and colleagues found that Gram''s iodine for plate flooding in place of hexadecyltrimethyl ammonium bromide or Congo red, gave a more rapid and highly discernable result [27]. However, plate-screening methods using dyes are not quantitative or sensitive enough due to poor correlation between enzyme activity and halo size. This has sparked the development of short cellooligosaccharides possessing modified reducing termini with chromogenic/fluorogenic groups due to achievement of higher sensitivity and quantification. Several examples such as fluorescein, resorufin and 4-methylumbelliferone are well-established [28-33]. A major limitation of the incorporation of fluorescent substrates into agar plates is the tendency for hydrolysis products to diffuse widely and therefore these kinds of compounds are not as readily used. Today, new substrates, 2-(2''-benzothiazolyl)-phenyl (BTP) cellooligosaccharides with degree of polymerization (DP) 2–4 (BTPG2–4) were synthesized for the screening of microbial cellulolytic activity in plate assays. The usefulness of the 2-(2''-benzothiazolyl)-phenyl substrates was shown during purification of the *Bacillus polymyxa* cellulolytic complex, which consists of at least three types of the enzymes: cellobiohydrolase, endo-β-D-glucanase and β-glucosidase [34]. Nonetheless, these methods are mainly limited to culturable cellulase-producing bacteria and the full cellulase-potential of the site (culturable and nonculturable microorganisms) is not being fully examined.

Researchers have now focused on the identification and exploitation of cellulase genes from unculturable microorganisms found in more extreme environments in hopes that the enzymes isolated will be novel and have specific applications in the biorefining industry due to a higher resistance to harsh environmental conditions. These enzymes may contribute to a decrease in the current cost of bioconversion of lignocellulose to ethanol by being more resistant to acids or bases used and by retaining activity at higher temperatures. To identify novel cellulases from all species present, culturable and

nonculturable in a swift manner, a metagenomic clone library should be created and then functionally screened; the key feature of this technique is the functional screening. Screening requires knowledge or rather an objective for the isolation of a specific enzyme with specific activity whether it be exoglucanases with activity on microcrystalline cellulose or endoglucanases with activity on soluble cellulose such as carboxymethyl cellulose (CMC). Depending on the objective different assays can be used to screen the recombinant proteins produced in *E. coli*. This is a quick and efficient method to screen a wide population which has been used recently to identify novel cellulase-producing bacteria from the rumen of buffalo and from pulp and paper mill effluent sediments by screening for crystalline and soluble cellulase activity [35,36]. Using different screening methods, a variety of cellulases with novel characteristics have been identified and are still being identified to date.

The isolation and identification of cellulases has been limited in the past to culturable microorganisms. However, recent advances in molecular techniques, such as the creation of metagenomic libraries will widen the pool of cellulolytic enzymes available for biofuel research. This approach will allow exploition of cellulases and related enzymes from otherwise unculturable microorganisms which may produce enzymes with novel characteristics.

Novel cellulase producing bacteria:

Isolation, screening and selection have favoured the discovery of several novel cellulase-producing bacteria from a wide variety of environments as previously discussed. Due to the vast diversity among bacteria the identification of novel cellulases remains a currently explored route to the improvement of biorefining industries. Here will be discussed briefly some of the new bacterial isolates and/or newly discovered and characterized cellulases, with potential use in the biorefining industry.

Recently, the bacterial strain B39, previously isolated from poultry manure compost in Taichung, Taiwan, was identified through 16S rRNA gene sequencing and phylogentic analysis to be a novel cellulose-degrading *Paenibacillus* sp. strain. A high-molecular weight (148 kDa) cellulase, possessing

both CMCase and Avicelase activities, was found to be secreted by this isolate into the media. At an optimal pH of 6.5, the CMCase activity of the newly isolated and purified cellulase was found to be approximately 2-fold greater than the activity on Avicel or filter paper and this cellulase was found to have maximum CMCase activity at 60°C, pH 6.5. Due to the promising thermostability of this enzyme, it has good potential for industrial use in the hydrolysis of soluble cellulose as well as activity on microcrystalline sources of cellulose [37]. Furthermore a novel cellulase-producing *Paenibacillus campinasensis* BL11 was isolated in 2006, from black liquor of brownstock at washing stage of the Kraft pulping process. This black liquor environment is strongly alkaline and therefore highly unfavourable to bacterial growth, isolation of a cellulase-degrading species from this environment provides plausibility that the enzymes produced by such a species could be tolerant to some of the harsh conditions used in the different pretreatments of lignocellulosic biomass. *P. campinasensis* BL11 is a thermophilic, spore-forming bacterium which was found to grow between 25 and 60°C over a wide range of pH. Optimal growth is around neutral pH, at 55°C. This isolate used a variety of saccharides (glucose, lactose, D(+)-mannose, D(+)-cellulobiose, L-arabinose etc.) and polysaccharides (starch, CMC, Avicel, xylan etc.) as carbon sourse in basal medium with organic nitrogen, and produced multiple extracellular saccharide-degrading enzymes including: a xylanase, two cellulases, a pectinase and a cyclodextrin glucanotransferase. The physiological properties of this strain and the vast number of free glycosyl hydrolases produced give this strain potential for use in the biorefining industry [38].

More recently, a thermostable cellulase was found in newly isolated *Bacillus subtilis* DR, extracted from a hot spring. The high temperature environment allowed for the production of a thermostable endocellulase CelDR with an optimum temperature at 50°C. It was found to retain 70% of its maximum activity (CMCase) at 75°C after incubation for 30 minutes. This strain offers a potentially more valuable thermostable enzyme for the biorefining industry due to extreme heat tolerance [39]. Cultivation of thermophiles offers several advantages, it reduces the risk of contamination, reduces viscosity thus making mixing easier, and leads to a high degree of substrate solubility while reducing the

cost of cooling. This is a greatly sought after property for cellulases in industrial applications like the bioconversion of lignocellulose. Also recently, a novel thermophilic, cellulolytic bacterium was isolated from swine waste and identified as *Brevibacillus* sp. strain JXL. It was found to use a broad spectrum of substrates such as crystalline cellulose, CMC, xylan, cellobiose, glucose and xylose. The crude extract of cellulolytic enzymes appeared to retain 50% of their activity after 1h at 100°C, making them highly thermostable [40]. Furthermore, a salt-activated endoglucanase was recently isolated from another *Bacillus* strain, alkaliphilic *Bacillus agaradhaerens* JAM-KU023 which was shown to have increased optimal thermostability from 50°C to 60°C with the addition of 0.2M NaCl and optimal pH range from 7-9.4 [41].

In addition, bacteria are capable of producing more complex protein structures supporting enzymes for the hydrolysis of cellulose, such as the cellulosome, xylosome and bifunctional or multifunctional enzymes which are currently gaining a lot of attention. If these enzymes can be recombinantly produced on mass or produced *in situ* by the bacterial strains naturally encoding them, then they may have great potential in improving the cost of hydrolysis for the production of biofuels by reducing the need for production of multiple enzymes for efficient hydrolysis. For example, a bifunctional endoglucanase/endoxylanase was isolated from *Cellulomonas flavigena* providing potential for use in different industrial processes such as biofuel production. This bifunctional enzyme was found to have optimum cellulase and xylanase activity at pH 6 and 9, respectively, with a general optimum temperature at 50°C [42]. Similarly, in 2007, a multifunctional enzyme was found to be produced by *Terendinibacter turnerae* T7902, which is a bacterial symbiont isolated from the wood-boring marine bivalve *Lydrodus pedicellatus*. This CelAB was found to have two catalytic and two carbohydrate-binding domains. It binds both cellulose and chitin and possesses cellobiohydrolase and beta-1,4(3) endoglucanase activity allowing it to degrade multiple complex polysaccharides. This enzyme is marginally acid-tolerant at an optimum pH of 6 and mesophilic with a temperature optimum of 42°C. Additionally, this enzyme was able to reduce viscosity of CMC approximately 40% after 25 minutes, displaying promising

characteristics for the biofuel industry [43]. All of these recently isolated enzymes and many more provide the framework needed to characterize and build highly efficient hydrolysis systems to be used in the biorefining industry. Isolation and characterisation of cellulase-producing bacteria will continue to be an important aspect of biofuel research.

Improvement of bacterial cellulases:

Despite the broad spectrum of cellulases being isolated, no single enzyme is completely suitable as it is, for the hydrolysis of cellulose in the biorefining industry. However, these enzymes offer a good starting point for the improvement of cellulases in steps towards enhancing the overall economics of biofuel production. Typically, the use of protein engineering technology has been directed towards the study of cellulase catalytic function. Mutagenesis has provided a means for studying the role of different amino acids within the catalytic domain. More recently, modifications to bacterial cellulases through the use of protein engineering is taking a stage in the production of efficient hydrolytic enzymes used in a broad scope of industries and includes targeting structural amino acids, beyond amino acids in the catalytic site. There are two major strategies for the improvement of a cellulase or cellulase component: 1) rational design and 2) directed evolution.

Rational design:

Rational design involves 1) choice of a suitable enzyme, 2) identification of the amino acid sites to be changed, based usually on a high resolution crystallographic structure, and 3) characterization of the mutants [44]. The use of rational design requires detailed knowledge of the protein structure: what makes the catalytic site active, a theoretical molecular structure-based model of the protein, and most ideally structure–function relationship. With at least part of this knowledge, modification of amino acid sequence can be achieved using site-directed mutagenesis, in some cases elements of secondary structure can be altered and even exchange of whole domains and/or generation of fusion proteins [44]. However, the vast

majority of enzymes do not have structural information available. Despite the fact that some target cellulases are well characterized, the molecular mutation required for the desired function cannot always be achieved [45]. To date, there are no general rules for site-directed mutagenesis strategies for the enhancement of cellulase activity and it therefore remains at present in a trial-like state. There is still limited knowledge about the properties of insoluble cellulosic substrates which differ based on pretreatment technologies; the interactions between cellulases and cellulose; and, the synergistic relationship among cellulase components. These factors hamper the ability of using rational design for improving bacterial cellulases for the biofuel industry.

To date, there are a few reports where site-directed mutagenesis was used to increase the catalytic activity of a bacterial cellulase. Mahadevan *et al.*, subjected the amino acids around the active site of endoglucanase Cel5A from *Thermotoga maritima* creating the N147E mutant which displayed 10% higher activity towards Avicel than the wild-type Cel5A [46]. The amino acids around the catlalytic-active center play a pivitol role in determing the rate of catalysis by stabilizing the carbonium ion intermediate. This group also showed a correlation between binding ability and the activity of the enzyme. By binding two CBDs, one from *Trichoderma reesei* and the other from *Clostridium stercorarium,* to Cel5A this CBD-engineered Cel5A displayed 14 to18-fold higher hydrolytic activity towards the crystalline cellulose Avicel [46]. In addition, the mutation of the conserved residue F476 involved in cellulose binding to Y476 of the CBD from Cel9A of *Thermobifida fusca* displayed 40% improved activity in assays with soluble and amorphous cellulose such as CMC and swollen cellulose. This was achieved through the integration of computer modeling with site-directed mutagenesis [47]. Furthermore, enzymatic activity was increased by 80% for a mutant Cel5Z endoglucanase of *Pectobacterium chrysanthemi* compared to the wild-type. However, this mutant enzyme was created by the use of a nonsense mutation which removed the C-terminal region creating a truncated Cel5Z containing 280 amino acids compared to the native Cel5Z which has 426 amino acids. Without the CBD this enzyme would not be efficient for hydrolysis of crystalline cellulose but could offer potential for

solubilised cellulose [48]. Likewise, the Cel5Z:: Ω mutant Cel5Z of *P. chrysanthemi* hydrolyzed CMC with 1.7-fold higher activity than the intact Cel5Z cellulase; this was due to the insertion of stop codon (Ω) that led to the removal of the C-terminal including CBD and linker region [49]. Similarly, a complex multifunctional enzyme Cel44C-Man26A secreted by *Paenibacillus polymyxa* GS01, was truncated from 1352 amino acids down to 549 amino acids. The truncated enzyme maintained cellulase, xylanase, mannanase and lichenase activities but on the contrary activity was not enhanced, however truncation allows the recombinant production of this multifunctional enzyme with more ease [50].

Furthermore, Baker and colleagues (2005) were able to design and mutate Tyr 245; an amino acid identified as a key residue interacting with a leaving group and related to reduced product inhibition of Cel5A of *Acidothermus cellulolyticus*. It was thus mutated to Gly and this was found to decrease the inhibition of the endoglucanase by cellobiose. Solubilized sugars were hydrolyzed 40% greater by the mutant Cel5A compared to the wild-type. Structural and kinetic studies correlated increased enzymatic activity to reduced product inhibition [51]. In addition, mutation of a single active-site cleft tyrosyl residue to a glycyl residue significantly changed the mixture of products released from phosphoric acid-swollen cellulose (PSC) from the catalytic domain of the endoglucanase-I from *A. cellulolyticus*. The percentage of glucose found in the product stream was approximately 40% greater for the Y245G mutant they created compared to the wild-type enzyme [52]. Bacterial cellulases improved by rational design are summarized in Table 1.

Classical chemical mutagenesis does not require knowledge of the protein structure and selection of desired traits becomes a guiding force for the development of improved enzymes. The maximum product yield of an endoglucanase from *Cellulomonas biazotea* deoxyglucose- mutant 51 was 1.5- to 2.5-fold more than was produced by the wild-type cells and was twice that reported by previous researchers on CMC [53]. Similarly, the highest productivity of *β*-glucosidase by a derived-mutant of *C. biazotea* was 2.5-fold more than that of the parent organism and the mutation stabilized the thermostability of the

enzyme [54]. This type of random mutation, although more crude, is indicative of the ideas directed evolution was based upon.

Directed Evolution:

Contrary to rational design, irrational design or directed evolution is an approach to non-informational protein engineering which utilizes the power of natural selection to evolve proteins and select for those with desired traits. Specifically, directed evolution requires the use of DNA techniques such as error-prone PCR (epPCR) and DNA shuffling to randomly generate a large library of gene variants. It has a great advantage over rational design because it is independent of enzyme structure and of the interactions between enzyme and substrate. Nonetheless, a major challenge of this method is developing a means to accurately evaluate the performance of mutants generated by recombinant DNA techniques and the selection of high-performance mutants. Screening methods typically include such tests as CMC agar with Congo red staining or the use of chromogenic or fluorogenic substrates, as previously mentioned. The more quantitative these methods are the greater chance of improving the directed evolution for improving bacterial cellulases. The success of directed evolution relies on a large library of gene variants, the larger, the greater the chance of mutants with desired properties.

The method of directed evolution was used to improve the thermal stability of *Clostridium cellulovorans* cellulosomal endoglucanase (EngB) *in vitro* by DNA recombination with non-cellulosomal endoglucanase EngD, based on the fact that the catalytic domains of both cellulase were highly homologous. The screening was done using CMC agar and staining with Congo red [55]. One of the mutants produced EngD with 7-fold higher thermostability and the authors suggest this to be due to a greater volume observed in the enzymes sidechains. Further, DNA shuffling was used to create a library of mutated endoglucanases from *B. subtilis* and was screened for increased catalytic activity. Interestingly, a bacterial surface display method was used to selectively screen for variants with improved activity on CMC agar with Congo red staining. This was done by fusing the genes with the ice nucleation

protein (Inp) the resulting fusion proteins would be displayed on the bacterial cell surface for easy

screening [56].

Furthermore, directed evolution using epPCR and family shuffling was used to successfully

increase thermal stability of β-D-glucosidases from *Paenibacillus polymyxa*, desired mutants were

screened using a chromogenic substrate [57,58]. Likewise, the catalytic activity of 1,4-β-D-glucan

glucohydrolase A from *Thermotoga neapolitana* was improved using epPCR to generate the gene variant

library [59]. While additionally, catalytic activity of a hyperthermostable β-glucosidase CelB from

Pyrococcus furiosus was improved by family shuffling. Catalytic activity was increased by 3- and 5-fold

compared to the wild-type; screening for successful mutants was accomplished by a chromogenic

substrate [60]. Finally, directed evolution of a glycosynthase from *Agrobacterium* sp. increased its

catalytic activity dramatically and expanded its substrate usage, the successful mutants were screened by

fluorogenic substrate [61]. Continued advancements in technology may increase the ease of using rational

design in attempts to improve cellulolytic enzymes. However, irrational design or random mutagenesis

will continue to be a dominant technique to alter cellulases because there is still much to be learned about

predicting protein structure and function. Some bacterial cellulases improved by rational design and

directed evolution are summarized in Table 1.

Table 1. A list of bacterial strains and cellulases or related enzymes from these microorganisms which
have been improved using rational design or directed evolution (Modified from Percival Zhang *et al.*,
2006).

Bacterial Strain	Enzyme	Property Altered	Method	Reference
Rational Design				
Acidothermus cellulolyticus	Endoglucanase	Type of products released	Site-directed mutagenesis	[52]
Acidothermus	Endoglucanase	Product	Site-directed	[51]

cellulolyticus		inhibition	mutagenesis	
Pectobacterium chrysanthemi	Endoglucanase	Activity	Nonsense mutagenesis	[48]
Pectobacterium chrysanthami	Endoglucanase	Activity	Insertional truncation	[49]
Thermobifida fusca	Processive Endoglucanase	Activity	Site-directed mutagenesis	[47]
Thermotoga maritime	Endoglucanase	Activity	Site-directed mutagenesis, CBD engineering	[46]
	Directed Evolution			
Agrobacterium sp.	Mutated α-glucosidase	Activity	epPCR	[61]
Bacillus subtilis	Endoglucanase	Activity	DNA shuffling	[56]
Clostridium cellulovorans	Endoglucanase	Thermal stability	Family shuffling	[55]
Paenibacillus polymyxa	β-D-glucosidase	Thermal stability	epPCR	[58]
Paenibacillus polymxa	β-D-glucosidase	Thermal stability	epPCR + family shuffling	[57]
Pyrococcus furiousus	α-glycosidase	Activity	Family shuffling	[60]
Thermotoga neapolitana	β-D-glucosidase	Activity	epPCR	[59]

Hemicellulase-producing bacteria and engineering hemicellulases:

Hemicellulose is the second most abundant renewable biomass, accounting for approximately 25-35% of lignocellulosic biomass and therefore bacterial enzymes involved in its degradation have also been the focus of several hemicellulase engineering studies using either rational design or directed evolution.

Rational design has been used to improve thermostability and functionality of several hemicellulases, however to date; rational design has not been successful in directly improving enzymatic activity. Four single, three double and one single disulphide bridge/s were constructed, using computer modeling, in the xylanase of *Bacillus circulans* and in GH-AA xylanase of *Thermobacillus xylanolyticus*. The half life of each mutant was 69°C for 120 mins and 70°C for 80 mins, respectively. Disulphide bonds increased thermostability 15°C over the wild-type but did not improve activity at elevated temperatures. The increased number of disulphide bonds was also shown to play a key role in thermostability [62,63]. Increasing enzyme thermostability is one step towards lowering biofuel production costs.

Moreover, increasing the versatility of a single hemicellulolytic enzyme could improve the hemicelluloytic properties and lower the amount of enzymes required for hydrolysis. Lu and Feng [64] created a bifunctional xylanase by creating an optimized flexible peptide linker between β-glucanase (Gl) of *Bacillus amyloliquefaciens* and the xylanase (Xyl) of *B. subtilis*. The catalytic efficiencies of Gl and Xyl moieties increased 304-426% and 82-143%, respectively, compared to an end-end fusion of Gl and Xyl that they have previously created [65]. Similarly, Fan and colleagues [66], also using a flexible peptide linker, created a multifunctional xylan-degrading enzyme. The xylanase domain of the xylanase XynZ from *Clostridium thermocellum*, was fused to a dual functional arabinofuranosidase/xylosidase (DeAFc; isolated from a compost starter mixture). The resulting trifunctional enzyme was more active in the hydrolysis of natural xylans and corn stover and retained pH, temperature optima, and, kinetics of the

parental enzymes [66]. Increasing the versatility of enzymes may increase activity through the synergistic action of fused enzymes and offer a greater production-cost savings.

Directed evolution, without the knowledge of enzyme structures, has been used to enhance thermostability, pH optima and specific activity of hemicellulases. A family shuffling technique referred to as degenerate oligonucleotide gene shuffling was created by Gibbs and colleagues [67], to reduce regeneration of unshuffled parental genes. One round of this technique was used after epPCR to generate a gene variant library and ultimately improve thermostability and pH optima (alkaline > 8.5 pH) of a family-11 xylanase (XynB) from *Dictyoglomus thermophilum* [67]. More recently, epPCR followed by 1 round of DNA shuffling was used to increase the melting temperature by 20°C for the xylanase XylA of *B. subtilis*. Screening of efficient variants was done using 1% oat spelt xylan and Congo red staining [68]. Consequently, epPCR has also been used to increase specific activity of xylanase Xys1 from *Streptomyces halstedii* JM8. By the random mutagenesis, two structural mutations were created (G133D and N148D) outside the catalytic centre. This slight structural change resulted in a 22-25% increase in specific activity of Xys1 towards xylan compared to the wild-type [69]. This study not only displays results towards creating more efficient enzymes for use in lignocellulosic biomass conversion; it also lends insight to key residues that are not directly involved in the catalytic site but play a indirect role in the active site function. Some bacterial hemicellulases improved by rational design and directed evolution are summarized in Table 2.

Rational design and directed evolution are helping to improve not only cellulases, but also hemicellulases by providing important insights about enzyme structure and function. Each contribution, no matter how large, is a step closer to improving lignocellulose biomass conversion.

Table 2. A list of bacterial strains and hemicellulases from these microorganisms which have been improved using rational design or directed evolution.

Bacterial strain	Enzyme	Property altered	Method	Reference
	Rational design			
Bacillus circulans	Xylanase	Thermostability	Site-directed mutagenesis	[62]
Bacillus amyloliquefaciens Bacillus subtilis	Bifunctional: xylanase-β-glucosidase	Substrate usage	Peptide linker fusion	[64]
Clostridium thermocellum Anaerobic digester	Trifunctional: xylanase, arabinofuranosidase / β-xylosidase	Substrate Usage	Peptide linker fusion	[66]
Thermobacillus xylanolyticus	GH-11 xylanase	Thermostability	Site-directed mutagenesis	[63]
	Directed evolution			
Bacillus subtilis	Xylanase XylA	Thermostability	epPCR, DNA shuffling	[68]
Dictyoglomus thermophilum	Xylanase XynB	Thermostability Alkalinity	DOGS, epPCR	[67]
Streptomyces halstedii	Xylanase Xys1	Activity	epPCR	[69]

Cellulosomes:

Cellulosomes are multienzyme complexes produced mainly by anaerobic bacteria, many from the class clostridia. However, evidence suggests the presence of cellulosomes in at least one aerobic bacterium and a few anaerobic fungi from species such as *Neocallimastix*, *Piromyces*, and *Orpinomyces* [70,71]. It is speculated that several other cellulolytic bacteria may also produce cellulosomes which have

yet to be described [40]. Production of cellulosomes by mainly anaerobic microorganisms is thought to be an evolutionary advantage which may counteract the low energy production by fermentation. Therefore, anaerobes produce this highly efficient multienzyme complex which allows for fine control over metabolic activities.

The cellulosome was first identified in 1983 from the anaerobic, thermophilic, spore-forming *Clostridium thermocellum* [72]. Unlike fungal cellulases, the *C. thermocellum* cellulase complex has very high activity on crystalline cellulose; this activity is termed "true cellulase activity" or Avicelase, characterized by its ability to completely solubilise crystalline forms of cellulose such as cotton and Avicel [73]. The cellulosome of *C. thermocellum* is commonly studied along with cellulosomes from the anaerobic mesophiles, *C. cellulolyticum* and *C. cellulovorans*. All cellulosomes share similar characteristics, they all contain a large distinct protein referred to as the scaffoldin which allows binding of the whole complex to microcrystalline cellulose via a nonspecific carbohydrate binding module (CBM). Also, the cellulosome scaffoldin expresses type I cohesins which allow binding of a wide variety of cellulolytic and hemicellulolytic enzymes within the complex via the expression of complementary type I dockerins on enzymes. Similarly, at the C-terminal the scaffoldin expresses type II cohesins which allow the binding of the cellulosomes to the cell through type II dockerins on surface layer-homology proteins (SLH) (Figure 1). The structure and function of bacterial cellulosomes have been reviewed several times elsewhere and will not be discussed in greater detail here [74-77].

The cellulosome eliminates the wasteful expenditure of energy of microorganisms continuously producing copious amounts of free enzymes along with which, the products get diluted in the bulk solution. There are several other advantages for microorganisms to naturally produce cellulosomes; specific characteristics of cellulosomes give rise to efficient cellulose hydrolysis. Firstly, synergism is optimized by the correct ratio between components, which is determined by the composition of the complex. Secondly, non-productive adsorption is avoided by the optimal spacing of the components

24

working together in the synergistic fashion. Thirdly, competitiveness in binding to a limited number of binding sites in the biomass surface is avoided by binding the whole complex to a single site through a strong binding domain with low specificity. Moreover, the close proximity of the cell to the substrate during enzymatic hydrolysis allows the close monitoring of inhibitory products and mediates the passage of cellobiose and cellodextrins into the cell for metabolism. Finally, a halt in hydrolysis on depletion of one structural type of cellulose at the site of adsorption is avoided by the presence of other enzymes with different specificity [74]. With all these advantages in mind and the available knowledge of these structures, cellulosomes may provide great potential for use in the biofuel industry.
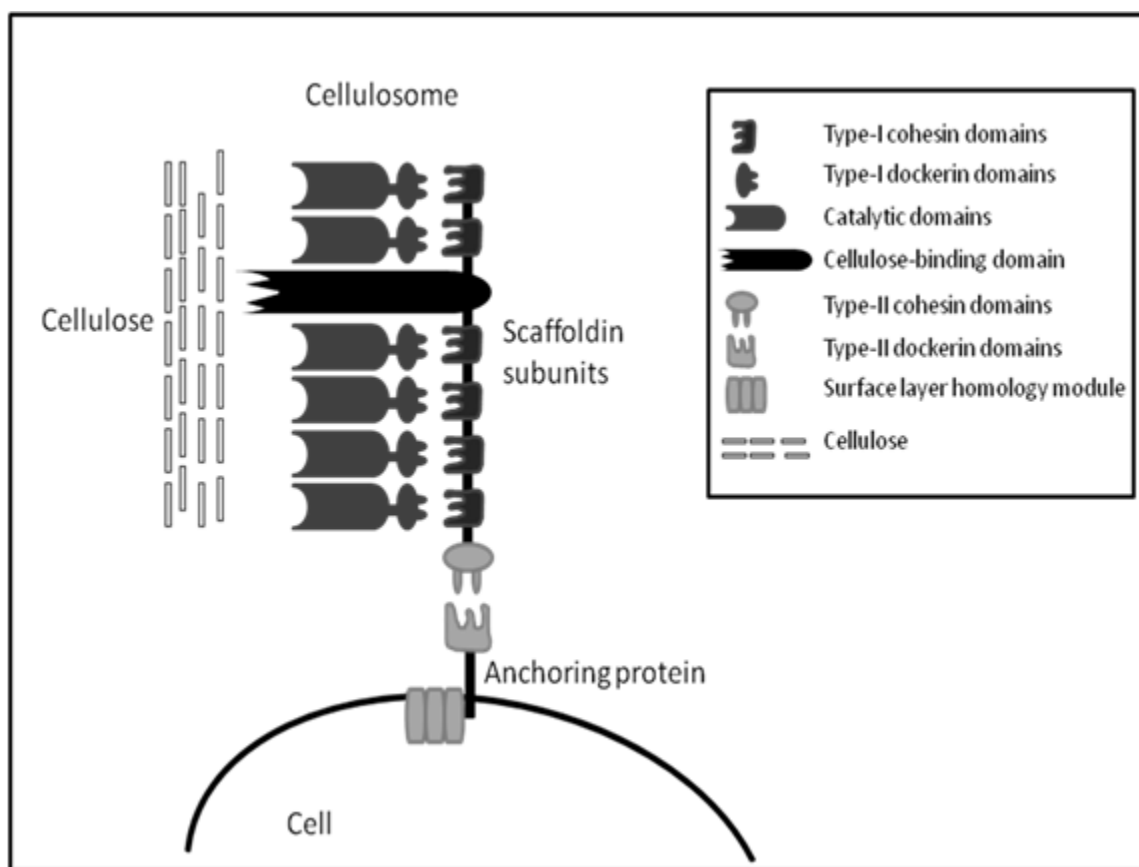
Figure 1. A simplified schematic of general cellulosome composition, connection with cell surface and interaction with substrate based on knowledge of *Clostridium* sp. cellulosomes. (Modified from Shoham *et al.* [74]).

Mini-cellulosome chimeras:

The complete genome sequence is available for *C. thermocellum* and the well known solvent-producing *C. acetobutylicum,* in the NCBI Genbank (NC_009012 and NC_003030, respectively). Additionally, several sequences for different cellulosomal scaffoldin are also now available. The sequencing of these genomes and various cellulosomal scaffoldin genes has opened the door for future enhancement of clostridia for cellulose hydrolysis and in some strains additionally fermentation. Researchers recognize the value of cellulosomes for the efficient hydrolysis of microcrystalline cellulose and have begun to focus research on creating designer cellulosomes for recombinant expression for industry and to advance our knowledge of true cellulolytic activity. Murashima and colleagues [55] created the first *in vitro* recombinant minicellulosomes with a specific function, using the scaffoldin structure of *C. cellulovoran* and the knowledge of cohesion-dockerin self assembly. The mini cellulosomes contained the enzymatic subunit EngB and the scaffolding unit, mini-CbpA, cellulose binding domain, a putative cell wall binding domain, and two cohesin units [55]. The full-length EngB containing the dockerin domain was expressed by *B. subtilis* WB800, which is deficient in eight extracellular proteases, to prevent the proteolytic cleavage of the enzymatic subunit between the catalytic and dockerin domains that was observed in previous attempts to express EngB with *Escherichia coli*. The mini-CbpA and cohesins were expressed by *E. coli.* This paved the way for *in vivo* synthesis of the EngB enzymatic subunit and mini-CbpA scaffolding unit by co-expression in *B. subtilis* [78]. Moreover, Perret and colleagues [79] created an enriched, highly specific cellulosome by cloning and overexpression of the Man5K gene in *C. cellulovorans*. Due to the high expression levels, Man5K was almost exclusively incorporated into the cellulosome resulting in a 20-fold increase in activity towards galactomannans and ultimately reducing specific activity on crystalline cellulose by 20% [79]. This is further evidence that the enzymatic composition of cellulosomes can be altered towards a specific activity.

Creating designer cellulosomes also allows us to examine properties of the cellulosome that may contribute to its efficiency. In „bifunctional" designer cellulosomes, two divergent cohesion-dockerin

devices from *C. thermocellum* and *C. cellulolyticum* of a CBD containing scaffoldin were compared to the function of similar minicellulosomes lacking CBD and free enzymes with and without CBDs. The result was higher cellulase activity on crystalline cellulose for minicellulosomes with CBD, however no apparent advantage over free enzymes on soluble substrate. The proximity of enzymes and the presence of CBD on the scaffoldin appear to contribute significantly and almost equally to the efficiency of the cellulosome on recalcitrant substrate [80]. Additionally, Fierobe and colleagues [81] used the same principle to create „trifunctional‟ designer cellulosomes with the addition of a third divergent cohesin-dockerin device from *Ruminococcus flavefaciens*. The trifunctional cellulosome chimera was found to be considerably more active than their previous bifunctional cellulosome, in addition to free enzymes. Their work also suggests that the cellulases from family-48 and -9 glycoside hydrolases are prominent and crucial for crystalline cellulose degradation. Also, co-operation and synergistic action between cellulases and hemicellulases of different organisms within designer cellulosomes, does exist and contribute to overall efficiency [81]. The largest designer cellulosome created using CbpA of *C. cellulovorans* contained four cohesins and was compared with activities of designer cellulosomes containing one and two cohesins. The incorporation of endoglucanase EngB and endoxylanase XynA enzymes in CbpA1234 again exemplified the importance of clustering for efficiency of cellulose degradation [82]. This research also provides evidence for the construction of more specific and larger designer cellulosomes with high activity.

Moreover, fungal cellulases were recently fused with dockerin sequences matching bacterial cohesins, and shown to be incorporated *in vivo* into mini bacterial cellulosomes alongside bacterial cellulases. These enzymes, despite species difference still showed increased synergy when bound in minicellulosomes further demonstrating the importance of synergy and enzyme proximity [83]. Similarly, the activities of free exoglucanases from *T. fusca* were compared to the activity of these enzymes incorporated into minicellulosomes. Incorporation showed a marked increase in cellulase activity due to increased synergy of the enzymes and close contact compared to free enzymes diluted in

the bulk solution [84]. These cellulosome chimeras offer an opportunity to take efficient cellulases or hemicellulases to further increase lignocellulosic hydrolysis.

In contrast to these studies, Mingardon and colleagues [83] deviated from designing cellulosome chimeras based on the general native structure of cellulosomes. Instead, they designed novel cellulosome chimeras which exhibited atypical geometries. Family-48 and -9 enzymes were modified to contain cohesins/dockerins and CBD"s, additional to the CBD and cohesins of the scaffoldin. This resulted in novel, oddly shaped cellulosomes. The number of protein-protein interactions within these complexes diminished the hydrolytic activity, due to the reduced mobility of the catalytic domains. Similarly, the presence of numerous CBD"s also restricted the activity and it appears that the native structure of the cellulosome is critical because it maximizes enzyme mobility [83].

The recent development of designer cellulosomes has unlocked key knowledge for the exploitation of cellulosomes in the bioconversion of lignocellulose. Designer cellulosomes offer a means to create specificity towards substrates and enhance enzyme activity with incorporation of efficient enzymes from a broad range of hosts. The next step is to find a means to develop cellulosome chimeras in a biologically and economically feasible manner.

The potential for cellulosome-producing *C. thermocellum*:

Due to the production of highly versatile cellulosomes and the anaerobic, thermophilic, ethanologenic nature, of *C. thermocellum*, it is an excellent candidate for consolidated bioprocessing (CBP). CBP features the production of cellulases and hemicellulases, hydrolysis of cellulose and hemicellulose, and, fermentation of hydrolysis products, all in one step. Using a strain such as *C. thermocellum* means less time for cooling and easy removal of ethanol at higher temperatures. It also means no addition of oxygen during the biorefining process and fermentation of glucose to produce ethanol and organic acids [85]. The compromise to using such a strain is the slow growth rate of anaerobic thermophiles; the possibility of spore-formation during biorefining; and, the fact that *C.*

thermocellum does not metabolize the 5-carbon sugars it produces during hydrolysis. Albeit, this strain is highly amenable to co-culture and co-culturing would allow growth with second or third party strains to enhance fermentation by the utilization of 5-carbon sugars as has been suggested [86,87].

Further, it has been observed that cellulase production in *C. thermocellum* is rapidly depressed by increasing concentrations of cellobiose [88]. However, the addition of exogenous β-glucosidase such as that purified from *Aspergillus niger*, can increase cellulosome activity up to 10-fold and offers a potential solution towards reducing cellulase inhibition [89]. In addition, the lower growth produced by anaerobic thermophiles can also be exploited as an advantage because it allows for prolonged ethanol production and less end-product inhibition to the hydrolysis enzymes due to an over abundance of enzymes.

An additional limiting factor to the exploitation of *C. thermocellum* for CBP for biomass conversion has been its recalcitrance to genetic modification. *C. thermocellum* has a strict restriction endonuclease system and is described as having a Dam+ phenotype [90,91]. However, several breakthrough DNA recombinant technologies are being developed for genetic engineering of the anaerobic clostridia. There are also DNA transformation protocols optimized specifically for *C. thermocellum* [92,93]. It has been shown that if DNA is Dam methylated it can provide protection to DNA from the restriction endonuclease system of *C. thermocellum*; therefore, if DNA is Dam methylated prior to electrotransformation a higher number of successful transconjugants should be seen [90]. A large number of plasmids have been developed for engineering thermophilic anaerobic bacteria [94-96]. The pIMK1 plasmid developed from the replicons of *C. acetobutylicum* and *Escherichia coli*, was used to successfully express kanamycin in *Thermoanaerobacterium saccharolyticum*, an anaerobic, thermophilic strain and close relative of *C. thermocellum*. Therefore this plasmid offers great potential framework for recombinant gene expression in *C. thermocellum* [97].

One final limiting factor for use of *C. thermocellum* in CBP is the inhibition of cell growth and metabolism by toxic by-products such as the production of acetic and lactic acids during fermentation to

produce ethanol. Blocking or knocking out genes involved in acetic and lactic acid production (e.g. acetate kinase and phosphotransacetylase), could help solve this problem. However, again the recalcitrance of clostridia to genetic modification has impeded this development. Nonetheless, a new technology has been developed been developed in 2007 for efficient gene knockout in clostridia: The ClosTron. The ClosTron utilizes Targetron technology which is a mobile group II intron originating from *Lactococcus lactis* L1 (LtrB intron). The LtrB intron allows a double-cross over event which is highly stable compared to previously used single-cross over events [98,99]. Successful transformants are selected based on erythromycin resistance and can be made in as short as 10 to 14 days for a variety of clostridia tested. Six knockout mutants of *C. acetobutylicum* were created and five knockout mutants of *C. difficile* were created, exceeding the number of mutants ever published for these species. Genes were also inactivated for the first time in *C. botulinum* and *C. sporogenes* [98]. These results make the ClosTron universally applicable to the clostridium genus and should therefore be of use in creating knockout mutants of *C. thermocellum*.

With recent great advancements in genetic technologies, overcoming the stumbling blocks of using *C. thermocellum* for a CBP process in the bioconversion of lignocellulosic biomass is a good concept with great potential. It may one day offer the most economically feasible means to create lignocellulosic derived ethanol.
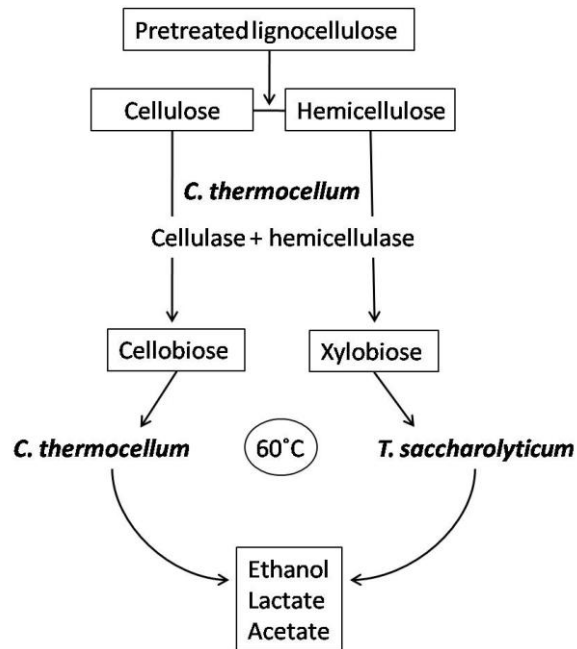
Figure 2. Simplified process using *C. thermocellum* and *T. saccharolyticum* in co-culture for ethanol production. *C. thermocellum* produces the cellulases and hemicellulases for hydrolysis of lignocelluloses to sugars such as cellobiose and xylobiose. In addition, *C. thermocellum* can utilize hexose sugars derived from celluloses to produce ethanol. While, the hemicelluloses derived pentoses can be utilized by *T. saccharolyticum*. *T. saccharolyticum* can also use glucose thus contributes to cellobiose reduction and is a good ethanol producer (modified from Demain *et al.* [85]).

Co-culture:

Bacterial co-cultures can offer a means to improve hydrolysis of cellulose as well as enhance product utilization and thus increase desirable fermentation products. *Clostridium thermocellum* has gained special interest for co-culture with organisms capable of fermenting pentose sugars to ethanol because C. *thermocellum* can only ferment hexose sugars. Hence, *C. thermocellum* has been co-cultivated with other anaerobic thermophilic clostridia or close relatives such as *Clostridium thermosaccharolyticum* (now classified as *Thermoanaerobacterium saccharolyticum*) [100-102],

Clostridium thermohydrosulfuricum [86,102,103], *Thermoanaerobacter ethanolicus* [104] and

Thermoanaerobium brockii [105]. These organisms can share a syntrophic relationship with *C. thermocellum* which exploits its cellulases and hemicellulases to hydrolyze cellulose to cellobiose and cellodextrans, and hemicelluloses to mainly xylobiose, arabinoxylans and xylooligosaccharides. *C. thermocellum* will then convert cellulose breakdown products to ethanol while the latter strains will utilize hemicellulose hydrolysis products to produce ethanol; this avoids the competition for substrates between species and maximizes product formation (Figure 2). The current challenge with this type of co-culture application is the increased production of by-products such as acetate and lactate which decrease ethanol production by slowing the growth rate of cells [106].

Developing bacterial co-cultures can be a tedious task. To establish a stable co-culture, media and growth requirements, such as temperature, atmosphere and carbon source, must be fine-tuned to permit equal growth of each strain. Stable co-cultures may not only depend on the media and growth requirements of each strain, but may also be controlled more specifically by metabolic interactions (i.e. syntrophic relationships or alternatively competition for substrates) and other interactions (i.e. growth promoting or growth inhibiting such as antibiotics). Criteria for structurally stable bacterial communities have been established, where 1) all the members must persist over more than 20 times subculturing and 2) the abundance ratio of members does not change even after subculturing. This is represented by reproducible growth, that is, subsequent subcultures without overgrowth or growth failure [107,108].

The alternative of bacterial co-culture would be to engineer one microorganism to complete an entire task from start to finish itself. In the case of *C. thermocellum*, this would mean metabolically engineering this strain to ferment pentose sugars in addition to hexose sugars. This is a difficult task as far as molecular engineering goes in clostridia due the recalcitrance of clostridia to genetic manipulation. Also to consider, if one could successfully engineer *C. thermocellum* to utilize pentose sugars, would this have an alternative effect on the ethanol yield produced from hexose sugars? Co-cultivation has

advantage because it reduces the number of exogenous elements produced by a single bacterial population and therefore reduces the chance of metabolic imbalance for host cells. Additionally, division of labor will simplify the optimization of each reaction pathway [109]. Although bacterial co-culture is not an uncommon concept, its use in the bioconversion of lignocellulosic biomass is still premature and offers great potential.

Closing comments:

Bacteria present an attractive potential for the exploitation of cellulases and hemicellulases due to their rapid growth rate, enzyme complexity and extreme habitat variability. The development of rapid and reliable methods for the screening of cellulases from microorganisms within inhospitable environments will allow a greater number of novel bacterial cellulases to be isolated with purpose for industrial use. None of the enzymes isolated to date, are fully resistant to the harsh environmental conditions used in the bioconversion process such as high temperature, acidic and or alkali pretreatments. However, these novel enzymes can be further engineered using available knowledge of enzyme structure and function through rational design. Or, they can be improved using random mutagenesis techniques with focus on selection of ideally augmented traits through directed evolution. Furthermore, novel or improved enzymes can be incorporated into designer minicellulosomes, which can further enhance the hydrolytic activity of individually efficient enzymes through synergy. Beyond free bacterial cellulases is the opportunity for whole cells in bacterial co-culture and the use of strains with multiple exploitable characteristics to reduce time and cost of current bioconversion processes. The future may hold great prospects for lignocellulosic biofuel; by combining our knowledge of excellent cellulolytic and hemicellulolytic systems such as the cellulosome of *C. thermocellum* with technologies such as directed evolution and co-culture, the future of lignocellulolytic biofuel looks potentially feasible.

References

1. Schneider SH. The Greenhouse Effect: Science and Policy. Science, 1989; 243: 771-781.

2. Annual Energy Outlook 1996 with Projections to 2015; U.S. Department of Energy, Energy Information Administration: DOE/EIA-0383(96), Washington, D.C., 1996.

3. Canadian Renewable Fuels Association: House of commons passes milestone biofuels bill, 2009. http://www.greenfuels.org/

4. US Environmental Protection Agency: EPA Proposes New Regulations for the National Renewable Fuel Standard Program for 2010 and Beyond**. Revised 26 May 2009.** http://www.epa.gov/OMS/renewablefuels/

5. AgMRC Renewable Energy Newsletter, October 2008. http://www.agmrc.org/ renewable_ energy/agmrc_renewable_energy_newsletter.cfm/brazils_ethanol_ industry_part_two_19

6. Greene et al. Growing energy. How biofuels can help end America"s oil dependence. Nat Res Def Council Rep. 2004;1-86.

7. Hill J, Nelson E, Tilman D, Polasky S, Tiffany D. Environmental, economic, and energetic costs and benefits of biodiesel and ethanol biofuels. Proc Natl Acad Sci USA. 2006;(314):1598-1600.

8. Wyman CE, Dale BE, Elander RT, Hotzapple M, Ladisch MR, Lee YY. Coordinated development of leading biomass pretreatment technologies. 2005;(96):1959-1966.

9. Philippidis GP, Smith TK, Wyman CE. Study of the enzymatic hydrolysis of cellulose for production of fuel ethanol by the simultaneous saccharification and fermentation process. Biotechnol Bioeng. 1993;41(9):846-853.

10. Lynd LR, van Zyl WH, McBride JE, Laser M. Consolidated bioprocessing of cellulosic biomass: an update. Curr Opin Biotechnol. 2005;16(5):577-583.

11. Henrissat B, Teeri TT, Warren RAJ. A scheme for designating enzymes that hydrolyse the polysaccharides in the cell walls of plants. FEBS Lett. 1998;(425):352-354.

12. Wood TM, McCrae SI. Synergism between enzymes involved in the solubilization of native cellulose. Adv Chem Ser. 1979;(181):181-209.

13. Rouvinen J, Bergfors T, Teeri TT, Knowles JKC, Jones TA. Three-dimensional structure of cellobiohydrolase II from *Trichoderma reesei*. Science. 1990;(249):380-386.

14. Divne C, Stahlberg J, Reinikainen T, Ruohonen L, Petterson G, Knowles JKC, Teeri T, Jones TA. The three dimensional structure of cellobiohydrolase I from *Trichoderma reesei* Science. 1994;(265):524-528.

15. Divne C, Stahlberg J, Teeri T, Jones TA. High-resolution crystal structures reveal how a cellulose chain is bound in the 50Å long tunnel of cellobiohydrolase I from *Trichoderma reesei* J Mol Biol. 1998;(275):309-325.

16. Davies GJ, Henrissat B. Structures and mechanisms of glycosyl hydrolases Structure. 1995;(3):853-859.

17. *CAZy* database - Carbohydrate-Active Enzymes database. Revised 22 June 2009. http://www.cazy.org/

18. Din N, Gilkes NR, Tekant B, Miller R C Jr, Warren RAJ, Kilburn, DG. Non-hydrolytic disruption of cellulose fibres by the binding domain of a bacterial cellulase. Bio/Technology. 1991;(9):1096-1099.

19. Johnson PE, Tomme P, Joshi MD, McIntosh LP. Interaction of soluble cellooligosaccharides with the N-terminal cellulose-binding domain of *Cellulomonas fimi* CenC. 2. NMR and ultraviolet absorption spectroscopy. Biochemistry.1996;(35):13895-13906.

20. Jervis EJ, Haynes CA, Kilburn DG. Surface diffusion of cellulases and their isolated binding domains on cellulose. J Biol Chem. 1997;272(38):24016-24023.

21. Sakon J, Irwin D, Wilson DB, Karplus PA. Structure and mechanism of endo/exocellulase E4 from *Thermomonospora fusca*. Nat Struct Biol. 1997;4(10):810-818.

22. Gal L, Gaudin C, Belaich A, Pages S, Tardif C, Belaich JP. CelG from *Clostridium cellulolyticum*: a multidomain endoglucanase acting efficiently on crystalline cellulose. J Bacteriol. 1997;179(21):6595-601.

23. Irwin D, Shin DH, Zhang S, Barr BK, Sakon J, Karplus PA, Wilson DB. Roles of the catalytic domain and two cellulose binding domains of *Thermomonospora fusca* E4 in cellulose hydrolysis. J Bacteriol. 1998;180(7):1709-1714.

24. Doi RH. Cellulases of mesophilic microorganisms: cellulosome and nocellulosome producers. Ann NY Acad Sci. 2008;(1125):267-279.

25. Rastogi G, Muppidi GL, Gurram RN, Adhikari A, Bischoff KM, Hughes SR, Apel WA, Bang SS, Dixon DJ, Sani RK. Isolation and characterization of cellulose-degrading bacteria from the deep subsurface of the Homestake gold mine, Lead, South Dakota, USA. J Ind Microbiol Biotechnol. 2009;36(4):585-598.

26. Hankin L, Anagnostakis S. Solid media containing carboxymethyl cellulose to detect CM celllulase activity of microorganisms. J Gen Microbiol. 1977;(98):109-115.

27. Kasana RC, Salwan R, Dhar H, Dutt S, Gulati A. A rapid and easy method for the detection of microbial cellulases on agar plates using gram's iodine. Curr Microbiol. 2008;57(5):503-507.

28. Wittrup KD, Bailey JE. A single-cell assay of β-galactosidase activity in *Saccharomyces cerevisiae*. Cytometry. 1988;(9):394–404.

29. Huang ZJ. Kinetic assay of fluorescein mono-β-D-galactoside hydrolysis by β-galactosidase: a front-face measurement for strongly absorbing fluorogenic substrates. Biochemistry. 1991;(930):8530–8534.

30. Eggertson MJ, Craig DB. β-Galactosidase assay using capillary electrophoresis laser-induced fluorescence detection and resorufin-β-D-galactopyranoside as substrate. Biomed Chromatogr. 1999;(13):516–519.

31. Irwin JA, Morrissey PE, Ryan JP, Walshe A, O'Neill SM, Carrington SD, Matthews E, Fitzpatrick E, Mulcahy G, Corfield AP, Dalton JP. Glycosidase activity in the excretory–secretory products of the liver fluke Fasciola hepatica. Parasitology. 2004;(129):465–472.

32. Xu H, Ewing AG. A rapid enzyme assay for β-galactosidase using optically gated sample introduction on a microfabricated chip. Anal Bioanal Chem. 2004;(378):1710–1715.

33. Fia G, Giovani G, Rosi I. Study of β-glucosidase production by wine-related yeasts during alcoholic fermentation. A new rapid fluorimetric method to determine enzymatic activity. J Appl Microbiol. 2005;(99):509–517.

34. Ivanen DR, Rongjina NL, Shishlyannikov SM, Litviakova GI, Isaeva-Ivanova LS, Shabalin KA, Kulminskaya AA. Novel precipitated fluorescent substrates for the screening of cellulolytic microorganisms. J Microbiol Methods. 2009;76(3):295-300.

35. Xu YQ, Duan CJ, Zhou QN, Tang JL, Feng JX. Cloning and identification of cellulase genes from uncultured microorganisms in pulp sediments from paper mill effluent. Wei Sheng Wu Xue Bao. 2006;46(5):783-788.

36. Duan CJ, Xian L, Zhao GC, Feng Y, Pang H, Bai XL, Tang JL, Ma QS, Feng JX. Isolation and partial characterization of novel genes encoding acidic cellulases from metagenomes of buffalo rumens. J Appl Microbiol. 2009;107(1):245-256.

37. Wang CM, Shyu CL, Ho SP, Chiou SH. Characterization of a novel thermophilic, cellulose-degrading bacterium Paenibacillus sp. strain B39. Lett Appl Microbiol. 2008;47(1):46-53.

38. Ko CH, Chen WL, Tsai CH, Jane WN, Liu CC, Tu J. Paenibacillus campinasensis BL11: a wood material-utilizing bacterial strain isolated from black liquor. Bioresour Technol. 2007;(14):2727-2733.

39. Li W, Zhang WW, Yang MM, Chen YL. Cloning of the thermostable cellulase gene from newly isolated Bacillus subtilis and its expression in Escherichia coli. Mol Biotechnol. 2008;(2):195-201.

40. Liang Y, Yesuf J, Schmitt S, Bender K, Bozzola J. Study of cellulases from a newly isolated thermophilic and cellulolytic Brevibacillus sp. strain JXL. J Ind Microbiol Biotechnol. 2009;36(7):961-970.

41. Hirasawa K, Uchimura K, Kashiwa M, Grant WD, Ito S, Kobayashi T, Horikoshi K. Salt-activated endoglucanase of a strain of alkaliphilic Bacillus agaradhaerens. Antonie Van Leeuwenhoek. 2006;89(2):211-219.

42. Pérez-Avalos O, Sánchez-Herrera LM, Salgado LM, Ponce-Noyola T. A bifunctional endoglucanase/endoxylanase from Cellulomonas flavigena with potential use in industrial processes at different pH. Curr Microbiol. 2008;57(1):39-44.

43. Ekborg NA, Morrill W, Burgoyne AM, Li L, Distel DL. CelAB, a multifunctional cellulase encoded by *Teredinibacter turnerae* T7902T, a culturable symbiont isolated from the wood-boring marine bivalve Lyrodus pedicellatus. Appl Environ Microbiol. 2007;73(23):7785-7788.

44. Percival Zhang YH, Himmel ME, Mielenz JR. Outlook for cellulase improvement: screening and selection strategies. Biotechnol Adv. 2006;24(5):452-81.

45. Arnold FH. Combinatorial and computational challenges for biocatalyst design. Nature. 2001;(409):253-257.

46. Mahadevan SA, Wi SG, Lee DS, Bae HJ. Site-directed mutagenesis and CBM engineering of Cel5A (*Thermotoga maritima*). FEMS Microbiol Lett. 2008;287(2):205-211.

47. Escovar-Kousen JM, Wilson D, Irwin D. Integration of computer modeling and initial studies of site-directed mutagenesis to improve cellulase activity on Cel9A from *Thermobifida fusca*. Appl Biochem Biotechnol. 2004;(113-116):287-297.

48. Lim WJ, Hong SY, An CL, Cho KM, Choi BR, Kim YK, An JM, Kang JM, Lee SM, Cho SJ, Kim H, Yun HD. Construction of minimum size cellulase (Cel5Z) from *Pectobacterium chrysanthemi* PY35 by removal of the C-terminal region. Appl Microbiol Biotechnol. 2005;68(1):46-52.

49. Park SR, Cho SJ, Kim MK, Ryu SK, Lim WJ, An CL, Hong SY, Kim JH, Kim H, Yun HD. Activity enhancement of Cel5Z from *Pectobacterium chrysanthemi* PY35 by removing C-terminal region. Biochem Biophys Res Commun. 2002;291(2):425-430.

50. Cho KM, Math RK, Hong SY, Asraful Islam SM, Kim JO, Hong SJ, Kim H, Yun HD. Changes in the activity of the multifunctional beta-glycosyl hydrolase (Cel44C-Man26A) from *Paenibacillus polymyxa* by removal of the C-terminal region to minimum size. Biotechnol Lett. 2008;30(6):1061-1068.

51. Baker JO, McCarley JR, Lovett R, Yu CH, Adney WS, Rignall TR, Vinzant TB, Decker SR, Sakon J, Himmel ME. Catalytically enhanced endocellulase Cel5A from *Acidothermus cellulolyticus*. Appl Biochem Biotechnol. 2005;(121-124):129-148.

52. Rignall TR, Baker JO, McCarter SL, Adney WS, Vinzant TB, Decker SR, Himmel ME. Effect of single active-site cleft mutation on product specificity in a thermostable bacterial cellulase. Appl Biochem Biotechnol. 2002;(98-100):383-94.

53. Rajoka MI, Ashraf Y, Khalid AM. Kinetic of improved production and carboxymethyl cellulose hydrolysis by an endo-glucanase from a derepressed mutant of *Cellulomonas biazotea*. Biotechnol Lett. 2004a;26(17):1329-1333.

54. Rajoka MI, Durrani IS, Khalid AM. Kinetics of improved production and thermostability of an intracellular beta-glucosidase from a mutant-derivative of *Cellulomonas biazotea*. Biotechnol Lett. 2004b;26(4):281-285.

55. Murashima K, Kosugi A, Doi RH. Thermostabilization of cellulosomal endoglucanase EngB from *Clostridium cellulovorans* by in vitro DNA recombination with non-cellulosomal endoglucanase EngD. Mol Microbiol. 2002;45(3):617-626.

56. Kim YS, Jung HC, Pan JG. Bacterial cell surface display of an enzyme library for selective screening of improved cellulase variants. Appl Environ Microbiol. 2000;66(2):788-793.

57. Arrizubieta MJ, Polaina J. Increased thermal resistance and modification of the catalytic properties of a beta-glucosidase by random mutagenesis and in vitro recombination. J Biol Chem. 2000;275(37):28843-28848.

58. Gonzalez-Blasco G, Sanz-Aparicio J, Gonzalez B, Hermoso JA, Polaina J. Directed evolution of beta -glucosidase A from *Paenibacillus polymyxa* to thermal resistance. J Biol Chem. 2000;275(18):13708-13712.

59. McCarthy JK, Uzelac A, Davis DF, Eveleigh DE. Improved catalytic efficiency and active site modification of 1,4-beta-D-glucan glucohydrolase A from *Thermotoga neapolitana* by directed evolution. J Biol Chem. 2004;279(12):11495-502.

60. Kaper T, Lebbink JH, Pouwels J, Kopp J, Schulz GE, van der Oost J, de Vos WM. Comparative structural analysis and substrate specificity engineering of the hyperthermostable beta-glucosidase CelB from *Pyrococcus furiosus*. Biochemistry. 2000;39(17):4963-70.

61. Kim YW, Lee SS, Warren RA, Withers SG. Directed evolution of a glycosynthase from *Agrobacterium* sp. increases its catalytic activity dramatically and expands its substrate repertoire. J Biol Chem. 2004;279(41):42787-42793.

62. Wakarchuk WW, Sung WL, Campbell RL, Cunningham A, Watson DC, Yaguchi M. Thermostabilization of the *Bacillus circulans* xylanase by the introduction of disulfide bonds. Protein Eng. 1994;7(11):1379-1386.

63. Paës G, O'Donohue MJ. Engineering increased thermostability in the thermostable GH-11 xylanase from *Thermobacillus xylanilyticus*. J Biotechnol. 2006;125(3):338-350.

64. Lu P, Feng MG. Bifunctional enhancement of a beta-glucanase-xylanase fusion enzyme by optimization of peptide linkers. Appl Microbiol Biotechnol. 2008;79(4):579-87.

65. Lu P, Feng MG, Li WF, Hu CX. Construction and characterization of a bifunctional fusion enzyme of *Bacillus*-sourced beta-glucanase and xylanase expressed in Escherichia coli. FEMS Microbiol Lett. 2006;261(2):224-230.

66. Fan Z, Werkman JR, Yuan L. Engineering of a multifunctional hemicellulase. Biotechnol Lett. 2009;31(5):751-757.

67. Gibbs MD, Nevalainen KM, Bergquist PL. Degenerate oligonucleotide gene shuffling (DOGS): a method for enhancing the frequency of recombination with family shuffling. Gene. 2001;271(1):13-20.

68. Ruller R, Deliberto L, Ferreira TL, Ward RJ. Thermostable variants of the recombinant xylanase A from *Bacillus subtilis* produced by directed evolution show reduced heat capacity changes. Proteins. 2008;70(4):1280-1293.

69. Díaz M, Rodriguez S, Fernández-Abalos JM, De Las Rivas J, Ruiz-Arribas A, Shnyrov VL, Santamaría RI. Single mutations of residues outside the active center of the xylanase Xys1 Delta from *Streptomyces halstedii* JM8 affect its activity. FEMS Microbiol Lett. 2004;240(2):237-243.

70. Fanutti C, Ponyi T, Black GW, Hazlewood GP, Gilbert HJ. The conserved noncatalytic 40-residue sequence in cellulases and hemicellulases from anaerobic fungi functions as a protein docking domain. J Biol Chem. 1995;270(49):29314-29322.

71. Li XL, Chen H, Ljungdahl LG. Two cellulases, CelA and CelC, from the polycentric anaerobic fungus *Orpinomyces* strain PC-2 contain N-terminal docking domains for a cellulase-hemicellulase complex. Appl Environ Microbiol. 1997;63(12):4721-4728.

72. Lamed R, Setter E, Kenig R, Bayer EA. The cellulosome: a discrete cell surface organelle of *Clostridium thermocellum* which exhibits separate antigenic, cellulose-binding and various cellulolytic activities. Biotechnol Bioeng. 1983;(13):163-181.

73. Johnson, E.A., Madia, A., Demain, A.L. Chemically defined minimal medium for the growth of the anaerobic cellulolytic thermophile *Clostridium thermocellum*. Appl Environ Microbiol.1981; (41):1060-1062.

74. Shoham Y, Lamed R, Bayer EA. The cellulosome concept as an efficient microbial strategy for the degradation of insoluble polysaccharides. Trends Microbiol. 1999;7(7):275-281.

75. Schwarz WH. The cellulosome and cellulose degradation by anaerobic bacteria. Appl Microbiol Biotechnol. 2001;56(5-6):634-649.

76. Bayer EA, Belaich JP, Shoham Y, Lamed R. The cellulosomes: multienzyme machines for degradation of plant cell wall polysaccharides. Annu Rev Microbiol. 2004;58:521-554.

77. Bayer EA, Lamed R, White BA, Flint HJ. From cellulosomes to cellulosomics. Chem Rec. 2008;8(6):364-377.

78. Cho HY, Yukawa H, Inui M, Doi RH, Wong SL. Production of minicellulosomes from *Clostridium cellulovorans* in *Bacillus subtilis* WB800. Appl Environ Microbiol. 2004;70(9):5704-7.

79. Perret S, Bélaich A, Fierobe HP, Bélaich JP, Tardif C. Towards designer cellulosomes in Clostridia: mannanase enrichment of the cellulosomes produced by *Clostridium cellulolyticum*. J Bacteriol. 2004;186(19):6544-6552.

80. Fierobe HP, Bayer EA, Tardif C, Czjzek M, Mechaly A, Bélaïch A, Lamed R, Shoham Y, Bélaïch JP. Degradation of cellulose substrates by cellulosome chimeras. Substrate targeting versus proximity of enzyme components. J Biol Chem. 2002;277(51):49621-49630.

81. Fierobe HP, Mingardon F, Mechaly A, Bélaïch A, Rincon MT, Pagès S, Lamed R, Tardif C, Bélaïch JP, Bayer EA. Action of designer cellulosomes on homogeneous versus complex substrates: controlled incorporation of three distinct enzymes into a defined trifunctional scaffoldin. J Biol Chem. 2005;280(16):16325-16334.

82. Cha J, Matsuoka S, Chan H, Yukawa H, Inui M, Doi RH. Effect of multiple copies of cohesins on cellulase and hemicellulase activities of *Clostridium cellulovorans* mini-cellulosomes. J Microbiol Biotechnol. 2007;17(11):1782-1788.

83. Mingardon F, Chanal A, López-Contreras AM, Dray C, Bayer EA, Fierobe HP. Incorporation of fungal cellulases in bacterial minicellulosomes yields viable, synergistically acting cellulolytic complexes. Appl Environ Microbiol. 2007;73(12):3822-3832.

84. Caspi J, Irwin D, Lamed R, Li Y, Fierobe HP, Wilson DB, Bayer EA. Conversion of *Thermobifida fusca* free exoglucanases into cellulosomal components: comparative impact on cellulose-degrading activity. J Biotechnol. 2008;135(4):351-357.

85. Demain AL, Newcomb M, Wu JHD. Cellulase, Clostridia, and Ethanol. Microbiol Mole Biol Rev. 2005;69(1):124–154.

86. Ng TK, Ben-Bassat A, Zeikus JG. Ethanol Production by Thermophilic Bacteria: Fermentation of Cellulosic Substrates by Cocultures of *Clostridium thermocellum* and *Clostridium thermohydrosulfuricum*. Appl Environ Microbiol. 1981;41(6):1337-1343.

87. Mori Y. Characterization of a Symbiotic Coculture of Clostridium thermohydrosulfuricum YM3 and *Clostridium thermocellum* YM4. Appl Environ Microbiol. 1990;56(1):37-42.

88. Brener D, Johnson BF. Relationship Between Substrate Concentration and Fermentation Product Ratios in *Clostridium thermocellum* Cultures. Appl Environ Microbiol. 1984;47(5):1126-1129.

89. Lamed R, Kenig R, Morgenstern E, Calzada JF, De Micheo F, Bayer EA. Efficient cellulose solubilisation by a combined cellulosome-β-glucosidase system. Appl Biochem Biotechnol. 1990;(27):173-183.

90. Klapatch TR, Demain AL, Lynd LR. Restriction endonuclease activity in *Clostridium thermocellum* and *Clostridium thermosaccharolyticum*. Appl Microbiol Biotechnol. 1996;45(1-2):127-131.

91. Ozkan M, Desai SG, Zhang Y, Stevenson DM, Beane J, White EA, Guerinot ML, Lynd LR. Characterization of 13 newly isolated strains of anaerobic, cellulolytic, thermophilic bacteria. J Ind Microbiol Biotechnol. 2001;27(5):275-280.

92. Tyurin MV, Sullivan CR, Lynd LR. Role of spontaneous current oscillations during high-efficiency electrotransformation of thermophilic anaerobes. Appl Environ Microbiol. 2005;71(12):8069-8076.

93. Tyurin MV, Desai SG, Lynd LR. Electrotransformation of *Clostridium thermocellum*. Appl Environ Microbiol. 2004;70(2):883-90.

94. Mather MW, Fee JA. Development of plasmid cloning vectors for *Thermus thermophilus* HB8: Expression of a heterologous, plasmid-borne kanamycin nucleotidyltransferase gene. Appl Environ Microbiol.v1992;(58):421-425.

95. Mai V, Wiegel J. Advances in development of a genetic system for *Thermoanaerobacterium* spp.: Expression of genes encoding hydrolytic enzymes, development of a second shuttle vector, and integration of genes into the chromosome. Appl Environ Microbiol. 2000;(660):4817-4821.

96. Moreno R, Zafra O, Cava F, Berenguer J. Development of a gene expression vector for *Thermus thermophilus* based on the promoter of the respiratory nitrate reductase. Plasmid. 2003;(490):2-8.

97. Mai V, Lorenz WW, Wiegel J. Transformation of *Thermoanaerobacterium* sp. strain JW/SL-YS485 with plasmid pIKM1 conferring kanamycin resistance. FEMS Microbiol Lett. 1997;(148):163-167.

98. Heap JT, Pennington OJ, Cartman ST, Carter GP, Minton NP. The ClosTron: a universal gene knock-out system for the genus *Clostridium*. J Microbiol Methods. 2007;70(3):452-464.

99. Shao L, Hu S, Yang Y, Gu Y, Chen J, Yang Y, Jiang W, Yang S. Targeted gene disruption by use of a group II intron (targetron) vector in *Clostridium acetobutylicum*. Cell Res. 2007;17(11):963-965.

100. Wang DIC, Avgerinos GC, Biocic I, Wang S-D, Fang H-Y. Ethanol from cellulosic biomass. Phil Trans R Soc London. 1983;(300):323-333

101. Venkateswaren S, Demain AL. The *Clostridium thermocellum-Clostridium thermosaccharolyticum* ethanol production process: nutritional studies and scale-down. Chem Eng Commun. 1986;(45):53-60.

102. Saddler JN, Chan MKH. Conversion of pretreated lignocellulosic substrates to ethanol by *Clostridium thermocellum* in mono- and coculture with *Clostridium thermosaccharolyticum* and *Clostridium thermohydrosulfuricum*. Can J Microbiol. 1985;(30):212–220.

103. Germain P, Toukourou F, Donaduzzi L. Ethanol production by anaerobic thermophilic bacteria: regulation of lactate dehydrogenase activity in *Clostridium thermohydrosulfuricum*. Appl Microbiol Biotechnol. 1986;(24):300–305.

104. Weigel J, Ljungdahl LG. Ethanol as fermentation product of extreme thermophilic, anaerobic bacteria, p. 117-127. *In* H. Dellweg (ed.),In Klassische Prozesse mit Neun Aussichten. Verlag Versuchs und Lehranstalt fuer Spiritusfabrikation and Fermentation Technologie im insituut fuer Gaerungsgewerbe und Biotechnologie, Berlin, germany.

105. Lamed R, Zeikus JG. Ethanol production by thermophilic bacteria: relationship between fermentation product yields of and catabolic activities in *Clostridium thermocellum* and *Thermoanaerobium brockii*. J Bacteriol. 1980;(144):569-578.

106. Herrero AA, Gomez RF, Roberts MF. 31P NMR studies of *Clostridium thermocellum*. Mechanism of end product inhibition by ethanol. J Biol Chem. 1985;260(12):7442-7451.

107. Kato S, Haruta S, Cui ZJ, Ishii M, Igarashi Y. Network relationships of bacteria in a stable mixed culture. Microb Ecol. 2008;56(3):403-11.

108. Kato S, Haruta S, Cui ZJ, Ishii M, Igarashi Y. Stable coexistence of five bacterial strains as a cellulose-degrading community. Appl Environ Microbiol. 2005;71(11):7099-106.

109. Brenner K, Lingchong Y, Arnold FH. Engineering microbial consortia: a new frontier in synthetic biology. Trends Biotechnol. 2008;66(9):483-489.

1.2 Lignin degradation in bacteria

Lignin is a complex polyphenol found in plants and is the most abundant aromatic in the biosphere; its complex structure makes it highly resistant to degradation (Ralph, 2005). Besides contributing to plant strength and resistance, it also acts as a barrier keeping microorganisms away from more readily degradable polysaccharides such as cellulose and hemicellulose (Monties and Fukushima, 2001). Primarily, studies have focused on degradation of lignin by the white-rot fungi and brown-rot fungi due to their ability to mineralize lignin (Sanchez, 2009). However, due to challenges in the genetic manipulation of fungi, as well as in protein expression from fungi there is currently no commercial biocatalytic process; thus, the prospects of using bacterial lignin-degrading genes has been gaining greater attention (Bugg *et al.*, 2011).

In 1988, the first reported lignin peroxidase in bacteria was characterized from extracellular extracts of *Streptomyces viridosporus* T7A (Ramachandra *et al.*, 1988). Moreover, using ^{14}C-labeled lignin *Rhodococcus* and *Nocardia* soil bacterium species also demonstrated breakdown of lignins (Zimmerman, 1990). Moving away from radioactive compounds, Ahmed *et al.* describe two spectrophotometric methods for measuring lignin degradation: one requires fluorescently labeled lignins, whereas the other involves the release of nitrated phenols from chemically nitrated lignins in a UV-vis assay (Ahmed *et al.*, 2010). Consequently, *Pseudomonas putida* mt-2 and *Rhodococcus jostti* RHA1 were confirmed for lignin degradation activities using these assays, as was revealed by the release of low molecular weight phenolic products. However, the lignin activities of these bacteria were lower in comparison to lignin degradation in white-rot fungi (*Phanerochaete chrysosporium*) (Ahmed *et al.*, 2010).

Additionally, potential abilities to break down lignin due to degradation of aromatics have been observed in bacteria isolated from the guts of termites. For example, *Rhodococcus erythropolis* was

isolated from the termite *Reticulitermes speratus* and was capable of degrading polycholorinated biphenyls, and displayed evidence for decomposition of lignin (Chung *et al.*, 1994). Moreover, *Burkholderia* and *Citrobacter* species were isolated from the lower gut of the termite *Coptotermes formosanus* displaying abilities to degrade aromatic compounds such as veratraldehyde and vanillin (Horazono *et al.*, 2003). All of the bacteria thus far suggested in the degradation of lignin or aromatics belong to three main classes: α-proteobacteria, γ-proteobacteria and actinomycetes (Bugg *et al.*, 2011).

Currently, the types of bacterial enzymes involved in lignin degradation are poorly understood. However, extracellular peroxidases have been found in bacteria such as *S. viridosporus* which can catalyze oxidative cleavage of β-aryl ether lignin models (Ramachandra *et al.*, 1988), while DyP-type peroxidase (DypB) was found to be expressed from *R. jostii* in the presence of Kraft lignin and ΔdypB mutants showed a significant reduction in Kraft lignin degradation (Ahmad *et al.*, 2011). In addition, a recent secretome analysis of *Thermobifida fusca* also revealed a dyp-type peroxidase (Adav *et al.*, 2010).

Furthermore, over 100 different bacterial laccases (multi-copper oxidase genes) were isolated from the DNA found in forest soils (Kellner *et al.*, 2008). Bacterial laccases may have considerable potential in lignin degradation as they are widespread in bacteria and are thought to play roles in pigmentation, sporulation and metal tolerance (Kellner *et al.*, 2008). There is little knowledge on the function of these widespread laccases however, some studies have shown that laccases can depolymerise lignin by oxidizing smaller molecules such as 2,20-azino-bis(3-ethylbenzothiazoline)-6-sulphonic acid (ABTS) and hydroxybenzotriazole (HBT).

Due to the resilience of bacteria to extreme environmental conditions such as acidity, alkalinity, salinity and drastic temperature changes, they represent possibly greater hosts for the exploitation of industrial lignases over fungal counterparts. For example, a copper-inducible laccase was found in Thermus thermophilus HB27 and was characterized as having a thermal inactivation half-life of greater than 14 h at 80°C (Miyazaki, 2005). Additionally, using anion exchange and gel filtration

chromatography a halotolerant-alkaline laccase was isolated from *Streptomyces psammoticus*; it was shown to retain 97% activity at pH 9.0 and NaCl tolerance up to 1.2 M (Niladevi *et al,* 2008).

To date, very few bacterial lignases have been characterized as mentioned here. Nonetheless, due to advances in the genetic manipulation of a wide variety of bacteria there remains great opportunity not only for isolation and expression of unique lignases, also further improvement of their catalytic activities.

References

Adav, S.S., Ng, C.S., Arulmani, M. and Sze, S.K. (2010) Quantitative iTRAQ secretome analysis of cellulolytic *Thermobifida fusca*. *J Proteome Res* 9, 3016-3024.

Ahmad, M., Roberts, J.N., Hardiman, E.M., Singh, R., Eltis, L.D. and Bugg TD. (2011) Identification of DypB from *Rhodococcus jostii* RHA1 as a lignin peroxidase. Biochemistry. **50**, 5096-107.

Ahmad, M., Taylor, C.R., Pink, D., Burton, K., Eastwood, D., Bending, G.R. and Bugg, T.D.H. (2010) Development of novel assays for lignin degradation: comparative analysis of bacterial and fungal lignin degraders. *Mol Biosystems* **6**, 815-821.

Bugg, T.D.H.. Ahmad, M., Hardiman, E.M. and Singh, R. (2011) The emerging role for bacteria in lignin degradation and bio-product formation. *Curr Opin Biotech* **22**, 394-400.

Chung, S.Y., Maeda, M., Song, E., Horikoshi, K. and Kudo, T. (1994) A Gram-positive polychlorinated biphenyl-degrading bacterium, *Rhodococcus erythropolis* strain TA421, isolated from a termite ecosystem. Biosci *Biotechnol Biochem* **58**, 2111-2113.

Harazono, K., Yamashita, N., Shinzato, N., Watanabe, Y., Fukatsu, T. and Kurane R. (2003) Isolation and characterization of aromaticdegrading micro-organisms from the gut of the lower termite Coptotermes formosanus. *Biosci Biotechnol Biochem* **67**, 889-892.

Kellner, H., Luis, P., Zimdars, B., Kiesel, B. and Buscot, F. (2008) Diversity of bacterial laccase-like multicopper oxidase genes in forest and grassland Cambisol soil samples. *Soil Biol Biochem* **40**, 638-648.

Monties B, Fukushima K (2001). Occurrence, function, and biosynthesis of lignins. In: Steinbüchel A, Hofrichter M, eds. Biopolymers, Vol 1 Lignin, humic substances, and coal. Weinheim, Germany: Wiley VCH, 1–64.

Miyazaki, K. (2005) A hyperthermophilic laccase from *Thermus thermophilus* HB27. *Extremophiles* **9**, 415-425.

Niladevi, K.N., Jacob, N., Prema, P. (2008) Evidence for a halotolerantalkaline laccase in *Streptomyces psammoticus*: Purification and characterization. *Process Biochem* **43**, 654-660.

Ralph K (2005). Actinomycetes and lignin degradation. Advances in Applied Microbiology, 58: 125–168.

Ramachandra, M., Crawford, D.L. and Hertel, G. (1988) Characterization of an extracellular lignin peroxidase of the lignocellulolytic actinomycete *Streptomyces viridosporus*. *Appl Environ Microbiol* **54**, 3057-3063.

Sanchez, C. (2009) Lignocellulosic residues: biodegradation and bioconversion by fungi. *Biotechnol Adv* **27**, 185-194.

Zimmermann, W. (1990) Degradation of lignin by bacteria. *J Biotechnol* **13**, 119-130.

1.3 Cellulase activities in biomass conversion: Measurement methods and comparison

Authors: Miranda Maki*, Mehdi Dashtban*, C. Mao, Kam Tin Leung, Wensheng Qin

Abstract

Cellulose, the major constituent of all plant materials and the most abundant organic molecule on the Earth, is a linear biopolymer of glucose molecules, connected by β-1,4-glycosidic bonds. Enzymatic hydrolysis of cellulose requires mixtures of hydrolytic enzymes including endoglucanases, exoglucanases (cellobiohydrolases) and β-glucosidases acting in a synergistic manner. In biopolymer hydrolysis studies, enzyme assay is an indispensable part. The most commonly used assays for the individual enzymes as well as total cellulase activity measurements, including their advantages and limitations are summarized in this review article. In addition, some novel approaches recently used for enzyme assays are summarized.

Keywords: Biofuel, Biomass, Bioconversion, Cellulase, and Cellulase Assays

Introduction

Many microorganisms including fungi and bacteria had been found to degrade cellulose and other plant cell wall fibres. In nature, degradation of cellulosic biomass is performed by mixtures of hydrolytic enzymes collectively known as cellulases. The cellulases include endo-acting (endoglucanases) and exo-acting (cellobiohydrolases, CBH) enzymes, which act in a synergistic manner in biomass-degrading microbes. The cellobiose and cellodextran products of exoglucanases and cellobiohydrolases are inhibitory to their activity. Thus, efficient cellulose hydrolysis requires the presence of β-glucosidases to cleave the final glycosidic bonds of cellobiose producing glucose (Dashtban et al. 2009; Maki et al. 2009).

Assays for determining cellulase activity have been classified differently over years of cellulase research. Sharrock (1988) grouped cellulase assays into two basic approaches: 1) determining the activities of individual cellulases (endoglucanases, exoglucanases, and β-glucosidases), and 2) measuring the total saccharifying activity of a crude cellulase system (Sharrock 1988). Whereas Zhang et al. (2006) classified all cellulase activity assays into three main groups: 1) assays in which the accumulation of products after hydrolysis were targeted, 2) assays in which the reduction in substrate quantity were monitored, and 3) assays in which the change in the physical properties of the substrate were measured (Zhang et al. 2006). Due to the complexity of cellulose-cellulase systems and differences between kinetic characteristics of initial hydrolysis reaction and the extended time, cellulase activity assays are either expressed based on the initial hydrolysis rate or using the end-point hydrolysis. The first one is preferred when measuring an individual cellulase activity in a short time; however, the last one is a method of choice for the total enzyme activity assay within a given time (Wu et al. 2006; Zhang et al. 2006).

Cellulase activity is mainly evaluated using a reducing sugar assay to measure the end products of cellulase hydrolysis activities. Thus, the results of such an assay are typically expressed as the hydrolysis capacity of the enzymes. There are several issues with this work: it cannot be easily expressed in a quantitative manner, lacks theoretical basis, and does not consider all effective factors, such as concentration of cellulose and cellulase, the hydrolysis time, the ratio of crystalline and amorphous cellulose, and the proportion between different individual components in the enzyme preparations (Wu et al. 2006). Researchers have mainly focused on improving methods for measurement of cellulase activity which have already been widely used. Developing new sufficient cellulase assays is hampered by the physical heterogeneity and limited enzyme-accessibility of cellulosic materials, and the complexity of cellulase enzyme systems (synergy and/or competition) (Zhang et al. 2006). Thus, an accurate and reproducible assay for the measurement of cellulase hydrolysis rate is still required (Wu et al. 2006).

In this review article, total cellulase activity by application of filter paper (filter paper assay, FPA) will be explained and then individual cellulase activities including endoglucanases, exoglucanases

and β-glucosidases will be discussed. Moreover, we will also summarize some novel approaches such as (1) quartz crystal microbalance, (2) miniaturized colorimetric assay, (3) automated FPA for the measurement of cellulase activity, (4) fluorescent microfibrils, and (5) amperometric cellobiose dehydrogenase biosensor. Figure 1 recaps the different cellulase assays discussed in the article. This review paper summarizes and compares past and present cellulase assaying techniques and suggests future directions important for the ever growing field of biofuel research.

1. Filter paper assay (FPase activity): total cellulase activity

To compare the efficacy of cellulase activity between microorganisms or their secreted enzymes, techniques for measuring total cellulase activity are required. The filter paper assay (FPA) is the key method for analysis of total cellulase activity. In 1976, the filter paper assay was developed by Mandels et al. (Mandels et al. 1976). The filter paper assay became widely used since 1984, when the Commission on Biotechnology of the International Union of Pure and Applied Chemistry (IUPAC) proposed a number of standard procedures for the measurement of cellulase activity. Traditionally, the filter paper assay uses a 1×6-cm strip of Whatman no. 1 filter paper, as the standard substrate because it is readily available and inexpensive (Coward-Kelly et al. 2003). This standard filter paper method has been reviewed by Ghose (Ghose 1987). The International Unit (IU) of filter paper activity (FPase) (FPU) is defined as the micromole of glucose equivalent liberated per minute of culture filtrate under assay conditions. Where assay conditions, refer to the conditions such as pH and temperature at which the enzymes are held at during the assay and depend largely on the properties of the enzyme, varying widely between cellulases and microorganisms. Reducing sugar is estimated as glucose by the Miller method. This assay is performed so that 0.5 mL of diluted enzymes releases about 2.0 mg of glucose equivalents in 60 min, as determined by the dinitrosalicylic acid (DNS) assay (Miller 1959; Wood and Bhat 1988).

The DNS reagent is used as a colorimetric method for the determination of reducing sugars, such as glucose. It contains sodium potassium tartrate, which decreases the tendency to dissolve oxygen by

increasing the ion concentration in the solution. Phenol increases the amount of color produced during the color developing reaction. Sodium bisulphite stabilizes the color obtained and reacts with any oxygen present in the buffer. Finally, an alkaline buffer is required for the redox reaction between DNS and glucose, or other reducing sugars. DNS will be added at the last step of the enzyme assay to stop the reaction. To promote full color development, samples have to be boiled vigorously and the absorbance of diluted samples will be read at 540 nm (Zhang et al. 2009). One disadvantage of using such a dye for quantification is that, some of the reducing sugars are degraded while the analysis is performed (Miller 1959).

There are several more concerns associated with using the filter paper assay to quantify total cellulase activity. Although the FPA is commonly used, it is also known for being non-reproducible. Difficulties arise from the preparation of the DNS reagent which is a tedious task requiring optimal mixing ratios of the different components. Additionally, DNS reagent requires appropriate temperature control to allow for proper colour development and colour stability (Miller 1959). Furthermore, it is known that the decomposition of sugars in the alkaline solution recommended by the IUPAC method causes an increase of (measured) enzyme activity to values higher than the actual ones (Gilman 1943). To summarize, it is time-consuming, labor-intensive and requires large quantities of reagents. It is also difficult to obtain adequate sensitivity and reproducibility when characterizing newly isolated cellulases using this method. Factors that affect sensitivity and reproducibility often result from the fact that most natural cellulase complexes tend to have a shortage of β-glucosidase activity (Breuil et al. 1986; Coward-Kelly et al. 2003).

Several methods have been developed to improve the filter paper assay for the evaluation of total cellulase activity. Nordmark et al. (2007) designed a modified method for the filter paper assay which requires the use of protein stabilizers. This method allows the sensitive measurement of cellulase activity below the level required for the detection of reducing sugars using the traditional filter paper assay. The traditional filter paper assay requires a fixed degree of conversion of substrate, i.e. a fixed amount (2 mg)

50

of glucose (based on reducing sugars measured by the DNS assay) released from 50 mg of filter paper within a fixed time (60 min). Because of the heterogeneous (amorphous/crystalline) nature of filter paper, reducing sugar yield during hydrolysis is not a linear function of the quantity of cellulase enzyme in the assay mixture (Zhang et al. 2009). To overcome this limitation, researchers usually measure two enzyme activities (slightly less than and slightly greater than 2.0 mg of Reducing Sugar Equivalents (RSE) in 1h). It is difficult to measure activities greater than 2.0 mg RSE in 1h for all cellulases because cellulases preparations typically have lower cellulase activity due to lower concentration. Protein stabilizers (such as bovine serum albumin, 1-2mg/ml) extended the enzyme reaction time thereby allowing a proportionate calculation of cellulase activities on natural cellulosic substrates to those obtained in the IUPAC assay (Nordmark et al. 2007).

Similarly, Coward-Kelly et al. (2003) found that the filter paper assay could be improved by adding supplemental β-glucosidase. If an organism or enzyme complex has low β-glucosidase activity a high amount of cellobiose will be produced resulting in a lowered or „false" absorbance reading for the DNS assay because it is not glucose. Adding supplemental β-glucosidase can help to overcome this issue. In this study, supplemental β-glucosidase increased the assay reading by 56%. They also tested the hypothesis that extended boiling time will improve the filter paper assay but failed to find any such benefit. A 5-min boiling time is sufficient; however, they suggest that the water bath boil vigorously to eliminate temperature excursions (Coward-Kelly et al. 2003).

Finally, downsizing the filter paper assay has also been developed as an improvement to the assay, allowing researchers to assay a large number of samples simultaneously. This has been achieved by reducing the volume of the reagents and substrate so the assay can be done in a 96-well microtitre plate. The overall enzymatic reaction volume was reduced from the IUPAC 1.5 mL standard to 60 μL. An office hole puncher was used to create small disks of filter paper substrate to fit perfectly in the wells. No significant difference was observed between the activities measured using the IUPAC filter paper assay compared to the minimized reactions in the microtitre plate (Xiao et al. 2004).

2. Endoglucanases activity: carboxymethyl cellulase activity (CMCase)

Endoglucanases (EG) can randomly hydrolyze internal glycosidic bonds in cellulose chains. EGs activities can be measured using a soluble cellulose derivative with a high degree of polymerization (DP) such as carboxymethyl cellulose (CMC). Carboxymethyl cellulase (CMCase) is mainly evaluated based on the procedure described by Mandels et al. (1976). In this method, CMCase activity is measured by determining reducing sugars released after 5 min of enzyme reaction with 0.5% CMC at pH 4.8 and 50 °C (Mandels et al. 1976). Also, one unit (IU) of EG is defined as the amount of enzyme that liberates 1 μmol of glucose per minute under assay conditions. Reducing sugar can be estimated by application of different methods such as high performance liquid chromatography (HPLC) (Fujita et al. 2002) or glucose oxidase/peroxidase reagent (Trinder 1969) or a colorimetric method such as the Somogyi-Nelson method which uses alkaline copper as an inorganic oxidant. Cupric ions (Cu (II)) accept electrons from the donating aldehyde groups of reducing sugars and reduce to Cu (I). In the second step, reduced Cu (I) ions will be oxidized back to Cu (II) using a chromogenic compound. The reduced chromogenic compound produces color which can be measured using a colorimeter and compared to standards prepared from reacting sugar solutions of known concentration, to determine the amount of reducing sugar present (Nelson 1944; Somogyi 1952).

Although CMC is commonly used as a substrate to quantify EG activity, there are several concerns associated with using CMC. It is known for being non-reproducible as it is only linear to about 12% hydrolysis (CMC to glucose) due to interference by substituents. In this case, substituted glucose units available in different CMCs are also accessible to cellulase which caused non-reproducibility. In addition, the quantity of reducing sugars produced and thus the unit values, will be highly affected by the particular type of CMC used in the assay (Eveleigh et al. 2009; Mandels et al. 1976). These difficulties arise from two important variable physical parameters of CMC: 1) the degree of substitution (DS), and 2) the degree of polymerization (DP) which will affect its solubility and viscosity, respectively. It is

recommended that a reducing sugar assay or viscosity assay should be limited to the first 2% hydrolysis of substrate when CMC is used as the substrate with DS=0.7, this is to ensure that only nonsubstituted glucose units are accessible to EG (Zhang et al. 2006). Additionally, the DP of CMC has an important role in determination of viscosity reduction. Therefore, to minimize the influence of some conditions such as pH and ionic strength on DP and thus viscosity, some substituted CMC substrates such as ionic CMC have to be avoided for determining EG activity. Whereas non-ionic substituted cellulose such as hydroxyethyl cellulose (HEC) is preferred (Guignard and Pilet 1976; Zhang et al. 2009).

EGs activities can be measured using dye, either by adding dye to soluble cellulose derivatives or by adding it to solid agar plates known as "zymograms". Remazol Brilliant Blue R and Ruthenium Red are two examples of dyes that have been used in CMC assays. Recently in a zymogram assay, Gram"s iodine has been used for a fast and easy detection of endoglucanase activity which makes a sharp and distinct zone around the cellulase producing microbial colonies in a bluish-black background within a short time (3-4 min) (Kasana et al. 2008). This method and other zymogram methods are applicable for screening of a large number of colonies. However, they do not provide a quantitative result for the enzyme activity due to the lacking of a linear relationship between halo zones and enzyme activity. Moreover, EGs activities can be measured using some other dyes by adding them to insoluble cellulose derivatives or substituting insoluble cellulose derivatives chemically to produce chromogenic CMC. Examples of these are Cibacron Blue 3GA (Ten et al. 2004) and chromogenic trinitrophenyl CMC (TNP-CMC) (Huang and Tang 1976), respectively.

3. **Exoglucanases activity: Avicellulases**

Cellobiohydrolases (exoglucanases) are classified as exo-acting based on the assumption that they all cleave β-1,4-glycosidic bonds from chain ends releasing cellobiose and some glucose molecules. Commercial Avicel (also called microcrystalline cellulose or hydrocellulose) is used for measuring

exoglucanase activity because it has a low degree of polymerisation of cellulose and it is relatively inaccessible to attack by endoglucanases despite some amorphous regions.

Enzymes which show relatively high activity on Avicel and little activity on CMC are identified as exoglucanases (Maki et al. 2009). However, Avicel contains some amorphous cellulose and soluble cellodextrans which can act as substrates for both exo- and endo-glucanases. There is no highly specific substrate to test exoglucanase activity in cellulase mixtures (Sharrock 1988; Wood and Bhat 1988).

Different assays have been reported for selection of exoglucanase activity, nevertheless all of these assays have some sort of limitations. Van Tilbeurgh and Claeyssens (1985) found that 4-methylumbelliferyl-β-D-lactoside was an effective substrate for assaying CBHI of *Trichoderma reesei*, where hydrolysis of this substrate yields lactose, phenol and 4-methylumbelliferone (a fluorescent signal molecule) as products. However, this substrate could not be used to determine CBHII activity of *T. reesei* thus it is not an effective representation of true exoglucanase activity for this strain (van Tilbeurgh et al. 1982; van Tilbeurgh et al. 1985).

Similarly, Deshpande et al. (1984) developed an assay for quantification of exoglucanase activity in the presence of endoglucanases and β-glucosidases (Deshpande et al. 1984). This assay is based on the following: exoglucanases specifically hydrolyze the aglyconic bond of *p*-nitrophenyl-β-D-cellobioside to yield cellobiose and *p*-nitrophenol; β-glucosidase activity is inhibited by adding D-glucono-1,5-δ-lactone (Holtzapple et al. 1990); and, the influence of exoglucanase hydrolysis activities must be quantified in the assay procedure in the presence of added purified endoglucanases. The limitations for this assay are that: (1) the CBHII activity cannot be measured using *p*-nitrophenyl-β-D-cellobioside, (2) the specific activity of the available purified endoglucanases may not be representative for all existing endoglucanases in the mixture, and (3) the product ratio from endoglucanase actions may be influenced by the presence of exoglucanases (Zhang et al. 2006).

Other less commonly used substrates for measuring or detecting exoglucanase activity for both bacteria and fungi include the following: PNP-β-D-cellobioside (Kohring et al. 1990), bacterial

microcrystalline cellulose (BMCC) (Caspi et al. 2008), and MU-β-D-cellobioside (MU-C) (Courty et al. 2005). Limitations of these substrates are not clearly defined.

4. β-glucosidases assay

β-glucosidase activity can be measured using various chromogenic and nonchromogenic substrates and are mainly evaluated based on the procedure of Kubicek (Kubicek 1982). In one chromogenic method, *p*-nitrophenol-β-glucoside (*p*NPG) is used as the substrate. The liberated *p*-nitrophenol will be measured in order to determine the hydrolysis rate in optimal temperature and pH. Reaction conditions such as temperature and pH of different β-glucosidases vary based on the enzyme (Table 1). *p*NPG as the substrate at the optimal concentration (usually 1-5 mM) will be added to an appropriate buffer with optimal pH, containing the enzyme and incubated at the optimal temperature. After 10-min incubation, the reaction will be stopped by adding 3 volumes of sodium tetraborate saturated solution, and then the absorbance will be read at 405 nm. One unit of β-glucosidase is defined as the amount of enzyme that liberates 1 μmol of *p*-nitrophenol per minute (Chandra et al. 2009). However, in the case of nonchromogenic substrates different methods can be used depending on the substrates. For example when oligo- or di-saccharides (such as cellobiose) are used as the substrates, the liberated glucose can be evaluated by the glucose oxidase (GOD) method with a commercial kit. Nevertheless, when the substrate is a polysaccharide, reducing sugars liberated will be measured by the 3,5-dinitrosalicylic acid (DNS) method. Using polysaccharides as the substrate to determine substrate specificity, the enzyme unit will be determined as the amount of enzyme required for the liberation of one micromole of glucose or reducing sugar per minute. Moreover, substrate specificity of enzymes can be determined using different substrates listed in Table 1 and applying the above mentioned methods.

β-glucosidase activity measurement using chromogenic substrates such as *p*NPG is a common technique used in many different studies (Bhatia et al. 2005; Daroit et al. 2008; Joo et al. 2009;

Karnchanatat et al. 2007; Korotkova et al. 2009; Murray et al. 2004; Tsukada et al. 2008; Yang et al. 2008; Yoon et al. 2008). However, correlation between β-glucosidase activity on the analog substrates (e.g. *p*NPG) and the natural substrate (e.g. cellobiose) is not clear. As a natural substrate, cellobiose has been used in β-glucosidase screening experiments using 96-well microtitre plates (McCarthy et al. 2004). However, this method is not preferred for screening of a large library of enzyme producing microorganisms due to its disadvantages such as being time-consuming and costly (Liu et al. 2009). Recently, several thermostable β-glucosidase (BGLA) mutants from *Paenibacillus polymyxa* have been identified using novel and fast combinatorial selection/screening approach. In this study a big mutant library including 100,000 clones were generated using error-prone PCR and cloned and expressed in *E. coli*. Approximately 30 thermostable β-glucosidase mutants have been identified in a two-step process using a natural substrate (cellobiose): 1) selection for mutants with adequate β-glucosidase activity; 2) screening for improved thermostability. In the first step, cells were grown on selection plates containing minimal growth medium plus cellobiose as the sole carbon source and thus, only cells expressing active β-glucosidase could grow on the medium. Colonies on the selection plate were duplicated using a nylon membrane and then incubated at 60 °C for 10 min to break the cells and release intracellular β-glucosidase. Also, heat treatment deactivated most of the β-glucosidase mutants and only thermostable β-glucosidase mutants will remain active and will be able to hydrolyse cellobiose to glucose on the screening plate. In the second step, the membrane was overlaid on the soft agar screening plate containing minimal medium with cellobiose as the sole carbon source. In addition to that, the medium contained an indicator strain of *E. coli* which was enabled to utilize glucose only (but not cellobiose). After incubation the growth of the indicator strain on the screening plate was used as an indicator to detect the clones expressing thermostable BGLA mutants. This screening method enable scientists to screen larger libraries within a shorter time. In this case, a thermotolerant mutant with 11-fold greater thermotolerance compared to the wild-type has been selected (Liu et al. 2009).

chromatography (Schwald et al. 1988). Recently a few novel assays with ease of operation and high reproducibility have been developed. Table 2 summarizes some cellulase assays using novel techniques.

One of the most recent novel assay methods uses a quartz crystal microbalance (QCM) piezoelectric-sensing technique, for measuring cellulase activity, and relates crystallinity of different substrates to the cellulase activity (Hu et al. 2009). The piezoelectric property of quartz crystal allows the production of an ultrasensitive mass balance. Changes in frequency of a quartz crystal can be used to measure viscosity and density changes in a solution used to incubate a given cellulose substrate, after enzymatic hydrolysis. The results can be used to quantify the enzyme activity. Here, the quantification of cellulase activity using QCM was closer to those results obtained by measuring the actual reducing sugars (IUPAC assay). QCM is advantageous to use because it is easier to implement by eliminating the need for colour development during the standard redox methods. It also allows for flexibility in the properties of substrates used. However, some difficulties arise from the thickness of substrates used as well as the sensitivity of the crystal.

Also recently, a miniaturized assay for the determination of total enzyme activity based on the colorimetric DNS method has been developed (King et al. 2009). In this study, the mini-assay proved useful for high-throughput bioprospecting of novel enzymes for biofuel production. Reducing sugar released from filter paper, Avicel, corn stalk, switchgrass, carboxymethylcellulose, and arabinoxylan were measured for a variety of fungal isolates and cellulase/hemicellulase activities comparable or greater than activities of the widely used wild-type *T. reesei* were observed. The enzyme extracts collected from cultures of biomass/substrate treated samples were aliquoted to 96-well microtitre plates and then DNS was added to stop the enzymatic reaction and measure the reducing sugars. The reagents were reduced producing the miniaturized assay (King et al. 2009). This miniaturized assay can be used not only for bioprospecting novel enzymes but also can be used to replace the traditional colorimetric cellulase assays

to measure and compare the activity of known cellulases. It is advantageous because it allows operators to reduce reagents, thereby reducing costs, aliquoting errors and ultimately the time for quantification.

Similarly, the possibility of complete automation of a cellulase assay was fully exemplified by Decker et al (2003). This group created an automated version of the traditional filter paper assay using a Cyberlabs C400 robotics deck equipped with customized incubation, reagent storage, and plate reading capabilities. The goal of such an automated assay was to reduce operator error during determination of cellulase activity and to reduce the amount of reagent usage as well as lower reagent disposal costs, while allowing for a high throughput of samples to be assayed. The maximum throughput of samples of the automated procedure is 84 enzymes per day. After the initial cost associated with the purchase of such a piece of equipment the high efficiency and low reagent usage will allow this technology to be successful, however at its current stage this automated assay is not comparable to the traditional FPA (Decker et al. 2003).

Furthermore, a more sensitive cellulase assay was developed using fluorescent microfibrils from bacterial cellulose prepared using DTAF (5-(4,6-dichlorotriazinyl) aminofluorescein) as a grafting agent. Fluorescent dyes such as DTAF which bear dichlorotriazinyl groups are known to react with hydroxyl groups of polysaccharides making DTAF a good candidate. A protocol to graft microfibrils with DTAF was developed which does not modify the physical integrity of the substrate. This grafted DTAF-cellulose was created by dissolving 10-70 mg of DTAF into 10 mL of a suspension containing 100 mg of cellulose microfibrils in 0.1 N NaOH. These mixtures were stirred at room temperature for 24 h. Cellulose digestion resulted in the release of fluorescent cellodextrins and reducing sugars. This method allowed for a comparison between the amount of released fluorescence and that of released reducing sugar from which one could differentiate between processive exo- and endo-cellulase activities. This research group also casted films of DTAF-grafted microfibrils to the bottom of microwell titre plates producing sensitive cellulase detection and allowing for possible automation. Sensitivity of detection can be increased by optimization of the grafting conditions which maximizes the quantity of soluble products.

59

The main advantages for using fluorescent microfibrils is it allows for measurement of nanomolar amounts of cellulase activity and it reduces the dependency on using substrates such as carboxymethyl cellulose which are far different from native cellulosic substrates. Cellulose microfibrils produced by algae and bacteria have been well characterized and shown to contain most of the structural and morphological characteristics of "real" cellulose materials. Being in a dispersed state, these cellulose microfibrils reduce cellulase-substrate accessibility problems (Helbert et al. 2003).

Moreover, Hildén et al. (2001), set out to create a faster, more convenient, yet equally reliable method for determining cellulase activities of a series of samples. They achieved this by using an amperometric redox polymer-based biosensor to determine the total concentration of soluble oligosaccharides. The biosensor was produced based on cellobiose dehydrogenase from *Phanerochaete chrysosporium* wired by a redox polymer. This newly applied method of measuring cellulase activity provides several advantages over traditional methods. Firstly, it is rapid, allowing analysis of a maximum 30 samples in an hour. In addition, the biosensor can be readily used without prior planning because it can be stored in water in flow injection analysis. Furthermore, the enzyme solution may be recovered after passing the electrode due to its non-destructive nature. Not to mention, no harmful chemicals, boiling or cooling is required with this method simplifying implementation. Finally, the precision of the method is equivalent to traditional methods such as the Somogyi-Nelson technique with high sensitivity detection to the same order of magnitude for cellobiose, cellotriose, and cellotetraose however without distinction of individually different sugars (Hilden et al. 2001).

Despite the newly emerging cellulase activity assays, the filter paper assay is still the most widely used method. Perhaps automation of the FPA will help researchers achieve reproducibility while reducing costs. However, biosensors are becoming more popular and may offer a similar promising solution to the evaluation of cellulase activity which will give results comparable to the direct measurement of reducing sugars via FPA.
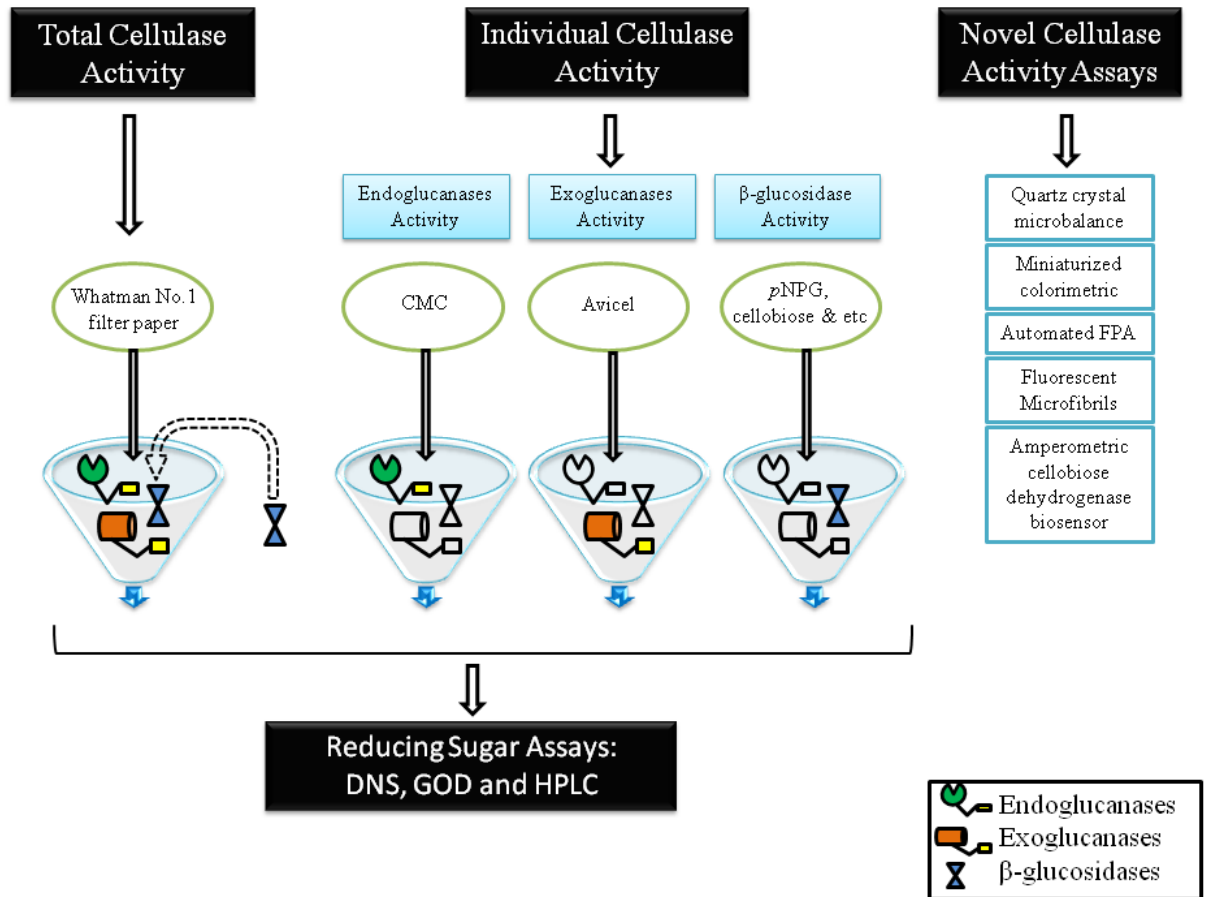
Figure 1. Different cellulase assays which are classified within two groups: 1) total cellulase activity, and 2) individual cellulase activity including endo-, exoglucanases and β-glucosidases. Filter paper assay can be improved by adding supplemental β-glucosidase which is indicated by the broken arrow. Released reducing sugars can be measured using different reducing sugar assay methods such as DNS (dinitrosalicylic acid), GOD (glucose oxidase), and HPLC. Recently a few novel assays with ease of operation and high reproducibility have been developed.

electrochemical techniques with enzymatic biosensors may potentially increase demands for investigation on cellulase assays to design high performance biosensing systems in terms of selectivity, sensitivity, reliability, durability, and low cost. An example of an amperometric biosensor with potential application in cellulase assays is the glucose-oxidase biosensor. The enzyme glucose oxidase is incorporated in the membrane of the electrode to detect glucose and ultimately relay glucose concentration. The glucose oxidase biosensor cannot detect small oligosaccharides such as cellobiose and cellotetraose which may be products of cellulase activity relating to endo- and exo- glucanases. However, the previously discussed cellobiose dehydrogenase containing amperometric biosensor is capable of measuring such products. For an accurate analysis of total cellulolytic activity we propose the production of a mixed enzyme membrane for biosensor detection. Combining glucose oxidase with an additional enzyme such as cellobiose dehydrogenase would allow the detection of all cellulose hydrolysis products.

References

Baronus, R., E. Ivanauskas and J. Kulys. 2003. "Computer simulation of the response of Amperometric Biosensors in stirred and non stirred solution." Nonlinear Analysis: Modeling and control 8:3-18.

Bhatia, Y., S. Mishra and V. S. Bisaria. 2005. "Purification and characterization of recombinant Escherichia coli-expressed Pichia etchellsii beta-glucosidase II with high hydrolytic activity on sophorose." Appl Microbiol Biotechnol 66(5):527-535.

Breuil, C., P. Mayers and J. N. Saddler. 1986. "Substrate conditions that influence the assays used for determining the beta-glucosidase activity of cellulolytic microorganisms." Biotechnol Bioeng 28(11):1653-1656.

Caspi, J., D. Irwin, R. Lamed, Y. Li, H. P. Fierobe, D. B. Wilson and E. A. Bayer. 2008. "Conversion of Thermobifida fusca free exoglucanases into cellulosomal components: comparative impact on cellulose-degrading activity." J Biotechnol 135(4):351-357.

Chandra, M., A. Kalra, P. K. Sharma and R. S. Sangwan. 2009. "Cellulase production by six Trichoderma spp. fermented on medicinal plant processings." J Ind Microbiol Biotechnol 36(4):605-609.

Chaubey, A. and B. D. Malhotra. 2002. "Mediated biosensors." Biosens Bioelectron 17(6-7):441-456. Courty, P. E., K. Pritsch, M. Schloter, A. Hartmann and J. Garbaye. 2005. "Activity profiling of ectomycorrhiza communities in two forest soils using multiple enzymatic tests." New Phytol 167(1):309-319.

Coward-Kelly, G., C. Aiello-Mazzari, S. Kim, C. Granda and M. Holtzapple. 2003. "Suggested improvements to the standard filter paper assay used to measure cellulase activity." Biotechnol Bioeng 82(6):745-749.

Daroit, D. J., A. Simonetti, P. F. Hertz and A. Brandelli. 2008. "Purification and characterization of an extracellular beta-glucosidase from Monascus purpureus." J Microbiol Biotechnol 18(5):933-941.

Dashtban, M., H. Schraft and W. Qin. 2009. "Fungal bioconversion of lignocellulosic residues; opportunities & perspectives." Int J Biol Sci 5(6):578-595.

Decker, S. R., W. S. Adney, E. Jennings, T. B. Vinzant and M. E. Himmel. 2003. "Automated filter paper assay for determination of cellulase activity." Appl Biochem Biotechnol 105 -108:689-703.

Deshpande, M. V, K. E. Eriksson and L. G. Pettersson. 1984. "An assay for selective determination of exo-1,4,-beta-glucanases in a mixture of cellulolytic enzymes." Anal Biochem 138:481-487.

Eveleigh, D. E., M. Mandels, R. Andreotti and C. Roche. 2009. "Measurement of saccharifying cellulase." Biotechnol Biofuels 2:21.

Fujita, Y., S. Takahashi, M. Ueda, A. Tanaka, H. Okada, Y. Morikawa, T. Kawaguchi, M. Arai, H. Fukuda and A. Kondo. 2002. "Direct and efficient production of ethanol from cellulosic material with a yeast strain displaying cellulolytic enzymes." Appl Environ Microbiol 68(10):5136-5141.

Ghose, T. K. . 1987. "Measurement of cellulase activities." Pure Appl Chem 59:257-268.

Gilman, H. 1943. "Organic Chemistry-Advanced Treatise.2nd ed." Wiley: New York.

Gopel, W. and P. Heiduschka. 1995. "Interface analysis in biosensor design." Biosens Bioelectron 10(9-10):853-883.

Guignard, R. and P-E. Pilet. 1976. "Viscosimetric determination of cellulase activity: critical analyses." Plant and Cell Physiology Vol. 17(5):899-908.

Helbert, W., H. Chanzy, T. L. Husum, M. Schulein and S. Ernst. 2003. "Fluorescent cellulose microfibrils as substrate for the detection of cellulase activity." Biomacromolecules 4(3):481-487.

Hilden, L., L. Eng, G. Johansson, S. E. Lindqvist and G. Pettersson. 2001. "An amperometric cellobiose dehydrogenase-based biosensor can be used for measurement of cellulase activity." Anal Biochem 290(2):245-250.

Holtzapple, M., M. Cognata, Y. Shu and C. Hendrickson. 1990. "Inhibition of Trichoderma reesei cellulase by sugars and solvents." Biotechnol Bioeng 36(3):275-287.

Hu, G., J. A. Heitmann and O. J. Rojas. 2009. "Quantification of Cellulase Activity Using the Quartz Crystal Microbalance Technique." Anal Chem.

Huang, J. S. and J. Tang. 1976. "Sensitive assay for cellulase and dextranase." Anal Biochem 73(2):369-377.

Joo, A. R., M. Jeya, K. M. Lee, W. I. Sim, J. S. Kim, I. W. Kim, Y. S. Kim, D. K. Oh, P. Gunasekaran and J. K. Lee. 2009. "Purification and characterization of a beta-1,4-glucosidase from a newly isolated strain of Fomitopsis pinicola." Appl Microbiol Biotechnol 83(2):285-294.

Karnchanatat, A., A. Petsom, P. Sangvanich, J. Piaphukiew, A. J. Whalley, C. D. Reynolds and P. Sihanonth. 2007. "Purification and biochemical characterization of an extracellular beta-glucosidase from the wood-decaying fungus Daldinia eschscholzii (Ehrenb.:Fr.) Rehm." FEMS Microbiol Lett 270(1):162-170.

Kasana, R. C., R. Salwan, H. Dhar, S. Dutt and A. Gulati. 2008. "A rapid and easy method for the detection of microbial cellulases on agar plates using gram's iodine." Curr Microbiol 57(5):503-507.

King, B. C., M. K. Donnelly, G. C. Bergstrom, L. P. Walker and D. M. Gibson. 2009. "An optimized microplate assay system for quantitative evaluation of plant cell wall-degrading enzyme activity of fungal culture extracts." Biotechnol Bioeng 102(4):1033-1044.

Kohring, S., J. Wiegel and F. Mayer. 1990. "Subunit Composition and Glycosidic Activities of the Cellulase Complex from Clostridium thermocellum JW20." Appl Environ Microbiol 56(12):3798-3804.

Korotkova, O. G., M. V. Semenova, V. V. Morozova, I. N. Zorov, L. M. Sokolova, T. M. Bubnova, O. N. Okunev and A. P. Sinitsyn. 2009. "Isolation and properties of fungal beta-glucosidases." Biochemistry (Mosc) 74(5):569-577.

Kubicek, C. P. 1982. "beta-Glucosidase excretion by Trichoderma pseudokoningii: correlation with cell wall bound beta-1.3-glucanase activities." Arch Microbiol 132(4):349-354.

Liu, W., J. Hong, D. R. Bevan and Y-H. P. Zhang. 2009. "Fast identification of thermostable beta-glucosidase mutants on cellobiose by a novel combinatorial selection/screening approach." Biotechnol Bioeng 103(6):1087-1094.

Maki, M., K. T. Leung and W. Qin. 2009. "The prospects of cellulase-producing bacteria for the bioconversion of lignocellulosic biomass." Int J Biol Sci 5(5):500-516.

Mandels, M., R. Andreotti and C. Roche. 1976. "Measurement of saccharifying cellulase." Biotechnol Bioeng Symp(6):21-33.

McCarthy, J. K., A. Uzelac, D. F. Davis and D. E. Eveleigh. 2004. "Improved catalytic efficiency and active site modification of 1,4-beta-D-glucan glucohydrolase A from Thermotoga neapolitana by directed evolution." J Biol Chem 279(12):11495-11502.

Miller, G. L. 1959. "Use of dinitrosalicylic acid reagent for determination of reducing sugar." Anal Chem 31:426-428.

Murray, P., N. Aro, C. Collins, A. Grassick, M. Penttila, M. Saloheimo and M. Tuohy. 2004. "Expression in Trichoderma reesei and characterisation of a thermostable family 3 beta-glucosidase from the moderately thermophilic fungus Talaromyces emersonii." Protein Expr Purif 38(2):248-257.

Nelson, N. . 1944. "A photometric adaptation of the Somogyi method for the determination of glucose." J Biol Chem 153:375-380.

Nordmark, T. S., A. Bakalinsky and M. H. Penner. 2007. "Measuring cellulase activity: application of the filter paper assay to low-activity enzyme preparations." Appl Biochem Biotechnol 137-140(1-12):131-139.

Schwald, W., M. Chan, C. Breuil and J. N. Saddler. 1988. "Comparison of HPLC and colorimetric methods for measuring cellulolytic activity." Appl Microbiol Biotechnol 28:398-403.

Sharrock, K. R. 1988. "Cellulase assay methods: a review." J Biochem Biophys Methods 17(2):81-105.

Somogyi, M. 1952. "Notes on sugar determination." J Biol Chem 195(1):19-23.

Ten, L. N., W. T. Im, M. K. Kim, M. S. Kang and S. T. Lee. 2004. "Development of a plate technique for screening of polysaccharide-degrading microorganisms by using a mixture of insoluble chromogenic substrates." J Microbiol Methods 56(3):375-382.

Trinder, P. 1969. "Determination of blood glucose using 4-amino phenazone as oxygen acceptor." J Clin Pathol 22(2):246.

Tsukada, T., K. Igarashi, S. Fushinobu and M. Samejima. 2008. "Role of subsite +1 residues in pH dependence and catalytic activity of the glycoside hydrolase family 1 beta-glucosidase BGL1A from the basidiomycete Phanerochaete chrysosporium." Biotechnol Bioeng 99(6):1295-1302.

van Tilbeurgh, H., M. Claeyssens and C. K. de Bruyne. 1982. "The use of 4-methylumbelliferyl and other chromophoric glycosides in the study of cellulolytic enzymes " FEBS Lett. 149:152 156.

van Tilbeurgh, H., G. Pettersson, R. Bhikabhai, H. De Boeck and M. Claeyssens. 1985. "Studies of the cellulolytic system of Trichoderma reesei QM 9414. Reaction specificity and thermodynamics of interactions of small substrates and ligands with the 1,4-beta-glucan cellobiohydrolase II." Eur J Biochem 148(2):329-334.

Wood, T. M. and K. M. Bhat. 1988. "Methods for measuring cellulase activities." Methods Enzymol 160:87-117.

Wu, B., P. J. Gao and Y. Zhao. 2006. "A new approach to measurement of saccharifying capacities of crude cellulase." BioResources 1(2):189-200.

Xiao, Z., R. Storms and A. Tsang. 2004. "Microplate-based filter paper assay to measure total cellulase activity." Biotechnol Bioeng 88(7):832-837.

Yang, S., Z. Jiang, Q. Yan and H. Zhu. 2008. "Characterization of a thermostable extracellular beta-glucosidase with activities of exoglucanase and transglycosylation from Paecilomyces thermophila." J Agric Food Chem 56(2):602-608.

Yoon, J. J., K. Y. Kim and C. J. Cha. 2008. "Purification and characterization of thermostable beta-glucosidase from the brown-rot basidiomycete Fomitopsis palustris grown on microcrystalline cellulose." J Microbiol 46(1):51-55.

Zhang, Y-H. P., J. Hong and X. Ye. 2009. "Cellulase assays." Methods Mol Biol 581:213-231.

Zhang, Y-H. P., M. E. Himmel and J. R. Mielenz. 2006. "Outlook for cellulase improvement: screening and selection strategies." Biotechnol Adv 24(5):452-481.

Chapter 2: Characterization of some efficient cellulase-producing bacteria isolated from paper mill sludges and organic fertilizers

Authors: Miranda L. Maki, Micheal Broere, Kam Tin Leung, Wensheng Qin,

2.1 Abstract

The wide variety of bacteria in the environment permits screening for more efficient cellulases to help overcome current challenges in biofuel production. This study focuses on the isolation of efficient cellulase producing bacteria found in organic fertilizers and paper mill sludges which can be considered for use in large scale biorefining. Pure isolate cultures were screened for cellulase activity. Six isolates: S1, S2, S3, S4, E2, and E4, produced halos greater in diameter than the positive control (*Cellulomonas xylanilytica*), suggesting high cellulase activities. A portion of the 16S rDNA genes of cellulase positive isolates were amplified and sequenced, then BLASTed to determine likely genera. Phylogenetic analysis revealed genera belonging to two major Phyla of Gram positive bacteria: Firmicutes and Actinobacteria. All isolates were tested for the visible degradation of filter paper; only isolates E2 and E4 (*Paenibacillus* species) were observed to completely break down filter paper within 72 and 96 h incubation, respectively, under limited oxygen condition. Thus E2 and E4 were selected for the FP assay for quantification of total cellulase activities. When grown in Dubois salts medium it was shown that 1% (w/v) CMC could induce total cellulase activities of 1652 ± 61 and 1456 ± 30 nM of glucose equivalents for E2 and E4, respectively. CMC could induce cellulase activities 8 and 5.6X greater than FP, therefore CMC represented a good inducing substrate for cellulase production. The genus *Paenibacillus* are known to contain some excellent cellulase producing strains, E2 and E4 displayed superior cellulase activities and represent excellent candidates for further cellulase analysis and characterization.

Keywords: Biodegradation, cellulase-producing bacteria, Firmicutes, Actinobacteria

2.2 Introduction

Increasing demand and the rising cost of fossil fuels, as well as a concern for global climate change have shifted global efforts to utilize renewable resources for the production of a „greener" energy replacement [1]. Lignocellulosic biomass, („plant biomass"), is a renewable, abundant and inexpensive resource for the bioconversion to biofuels and bioproducts. It is comprised of mainly cellulose, a homologous polymer of glucose molecules connected by β-1,4 linkages (the most abundant organic polymer in the world). It also contains some hemicellulose (a heterologous polymer of 5- and 6-carbon sugars) and even less so lignin (a complex aromatic polymer). Known as abundant, there are a great many sources to derive lignocellulosic biomass from such as municipal waste, agricultural residues, forestry or pulp and paper excesses, and, energy crops (i.e. Switchgrass) [2].

Several microorganisms including both bacteria and fungi have been found to produce a variety of cellulases for the degradation of cellulose. Primarily, cellulases are classified into three main groups: the exoglucanases, endoglucanases (cleaving β-1,4-glycosidic bonds from chain ends and internally within chains, respectively) and β-glucosidases (cleave the final β-1,4 linkage of cellobiose or small polysaccharides) [3]. Bacteria and fungi have been found to produce and secrete these enzymes freely in solution; however, some microorganisms have also been found to produce cell-bound enzymes and multi-protein complexes expressing cellulases and hemicellulases called cellulosomes. The cellulosome was first discovered in 1983 from the anaerobic, thermophilic spore-forming *Clostridium thermocellum* [4].

One major obstacle facing the development of lignocellulosic biofuels is the cellulose hydrolysis stage. Generally speaking, there is a lack of microorganisms which can produce sufficient amounts of all three types of cellulases to efficiently breakdown crystalline cellulose to glucose. Moreover, the biorefining process remains economically unfeasible due to a lack of biocatalysts that can overcome costly hurdles such as cooling from high temperature, pumping of oxygen/stirring, and, neutralization from acidic or basic pH. The extreme environmental resistance of bacteria permits screening and isolation of novel cellulases to help overcome these challenges.

Although molecular engineering is leading researchers in the field of biorefining towards developing microorganisms which can produce a greater number of more efficient cellulases, the traditional microbiological technique of isolation still plays an important role. New isolates lay the foundation for molecular engineering strategies, perhaps a new cellulase-degrading strain may represent a good host or framework to further improve or add new enzyme-encoding genes for further improvement. Similarly, a cellulase produced by an isolate may be more efficient and whose gene may be worth cloning and introducing to an already good industrial cellulase producer to further improve its cellulose-degrading repertoire. Each small step will make biorefining and ethanol production more economically feasible and will help take reliance off of petroleum based fuels and allow progression towards a more renewable fuel source.

In this study, several efficient aerobic cellulase-producing microorganisms were isolated from different pulp and paper mill sludges and one commercial microbially enhanced soil amendment sample. The purpose was to identify and characterize those isolates displaying the greatest cellulase activity for the possible use in large scale biorefining.

2.3 Materials & Methods

2.3.1. Lignocellulosic samples and media used

The lignocellulosic samples for isolation of cellulase-producing bacteria were obtained from a few sources. Two samples were obtained from the area of Red Rock, Ontario, Canada and were labeled B (black-coloured solid sludge) and W (wood-like solid sludge). Both B and W were dry, aged waste products leftover from pulp and paper mill processing. The exact stage and treatment of the sludge is not known. Additionally, a sludge material (S) produced from the kraft processing of fine paper was obtained from a paper mill in Thunder Bay, Ontario, Canada. The center of the sludge sample displayed microbial

70

activity which was observed by a change in colour of the sludge from white to grey. Finally, a commercial fertilizer was analyzed which is called Efficient Microorganism Dust (RedRock, ON) (E).

The growth media used in the experiments include R2A agar (0.5g l^{-1} yeast extract, 0.5g l^{-1} protease peptone, 0.5g l^{-1} casamino acids, 0.5g l^{-1} glucose, 0.5g l^{-1} soluble starch, 0.3g l^{-1} dipotassium phosphate, 0.5g l^{-1} magnesium sulfate 7H$_2$O, 0.3g l^{-1} sodium pyruvate, 15.0g l-1 agar), LB liquid media (10.0g l^{-1} peptone, 5.0g l^{-1} yeast extract, 5.0g l^{-1} NaCl), and carboxymethyl cellulose agar (0.5g CMC, 0.1g NaNO$_3$, 0.1g K$_2$HPO$_4$, 0.1g KCl, 0.05g MgSO$_4$, 0.05g yeast extract, 1.5g agar, per 100 ml ddH$_2$O, pH 7.4) [5].

2.3.2. Isolation of bacteria from lignocellulosic samples using R2A

To isolate bacteria, 1 g of each sample was suspended in 20 ml of sterile potassium phosphate buffer solution (PPB) by vortexing for 2 min on maximum speed. Following, a 10X serial dilution of the suspension was made in PBS. Thereafter, 200μl of each dilution in the series was spread onto the surface of R2A agar using the standard spread plate technique. All plates were incubated at 28°C for 24 h before sampling and then they were incubated for an additional 48 and 72 h to allow growth of slower growing microorganisms for further sampling. From the growth observed over 24, 48 and 72 h, various colonies were selected based on their morphology, size and colour. The colonies selected were then streaked out on separate R2A plates to ensure purity. Colonies were further subcultured on R2A if more purification was required. After purification, the cultures were compared visually to eliminate those of similar size, morphology and colour. The plates were then photographed and described for a database (database not shown here).

2.3.3. Screening for carboxymethyl cellulose activity

Isolates were grown in 10 ml of LB broth for 24 h, shaking at 28°C, slower growing isolates were left to incubate for an additional 48 h. The positive control used was *Cellulomonas xylanilytica*. This strain was also grown in the LB medium; however it required incubation for a 5 day period using the same growth

conditions. The incubation of *C. xylanilytica* was determined based on the colony growth rate which requires four days incubation before colony development, the 5th day representing 48h colony presence. The negative control used was *Escherichia coli* JM109, also grown in LB broth overnight; however, it grows at 37°C for 18 h. All resulting broth cultures (isolates, positive control and negative control) were tested for cellulase activity via the Gram˝siodine method [5]. In this method, 5μl of each broth culture were singly dropped onto a plastic Petri dish containing carboxymethyl cellulose (CMC) agar and then incubated for 48 hours at 28°C. The positive control was incubated for an additional 60 h longer than the isolates and negative control due to its slow growth rate. After the allotted growth time, the CMC agar plates with the isolates and controls were stained at room temperature with Grams iodine solution (2.0g KI and 1.0g I, per 300ml ddH$_2$O) to visualize the cellulase activity. This solution stains the agar containing CMC brown and leaves areas without CMC clear, described here as halos. The appearance of clear halos around the drops confirms cellulase activity by the bacteria. Each plate was flooded completely with approximately 5 ml of the Grams iodine solution using a Pasteur pipette. The plates were allowed to sit for 5 minutes until the dye settled into the media and then they were photographed for a database not shown here. The cellulase positive isolates were then re-grown in LB broth and drop plated onto smaller CMC plates (50mm × 9mm) using the same techniques and conditions previously described, shown in Figure 1. From the new, smaller, CMC plates, the halo diameters were measured using a ruler for a semi-qualitative comparison of cellulase activity among the isolates. The halo measurement is used to relate cellulase activity to bacteria position on the phylogenetic tree as shown in Figure 2.

2.3.4. DNA isolation and 16S rDNA amplification

The cellulase producing isolates as well as the positive control were grown up in LB broth for 24 h at 28°C. DNA was isolated from each isolate broth culture using the Fungi/Yeast Genomic DNA Isolation Kit from Norgen Biotek Corporation, Canada. The resulting isolated DNA was used as a template in a PCR reaction to amplify a region of the 16S rDNA. Universal primers designed within conserved regions

of the 16S rDNA for Eubacteria were used: HAD-1 (5′-GACTCCTACGGGAGGCAGCAGT) and

E1115R (5′-AGGGTTGCGCTCGTTGCGGG), they amplify approximately a 796 bp fragment [6]. The

PCR reaction mixtures contained 10 ng of genomic DNA individually from each positive isolate, 10 pmol

of both forward and reverse primers, 10x Taq buffer with KCl, 25 mmol l^{-1} MgCl$_2$, 0.2 mmol

deoxynucleoside triphosphate, and 5 U Taq DNA polymerase per 50 µl reaction. The PCR program was

as follows: primary denaturation 3 minutes at 95°C, followed by 33 amplification cycles consisting of

denaturing at 95°C for 1 minute, annealing for 1 minute at 63°C, and extension at 72°C for 1 minute,

upon completion of 33 amplification cycles a final extension step was done at 72°C for 10 minutes. The

PCR products were then viewed on a 1% agarose gel to confirm size, quantity and purity. Then, PCR

products were sequenced using standard run modules on the ABI 3730xl automatic sequencer (Eurofins

MWG Operon, Canada).

2.3.5 Isolate Identification and Relatedness

Sequencing results were individually inputted online into the nucleotide blast tool through the NCBI

database (http://blast.ncbi.nlm.nih.gov/) to identify the possible genera of the isolates. Sequencing results

of the isolates and positive control were also inputted into a sequence alignment program called ClustalX

to determine the phylogenetic relatedness of the different species. They were aligned using the UPGMA

algorithm, which considers the rate of evolution to be constant between species, to develop a phylogenetic

tree based on sequence homology. The resulting alignment was opened into a program called TreeView

which allowed the phylogenetic tree to be viewed.

2.3.6. Qualitative filter paper activity

Isolates displaying cellulase activity on the CMC plates were further screened for quality of cellulase

activity by transferring 100 µl of an overnight culture to 5 mL of Dubois salts media (K2PO4 1g l^{-1}, KCl

0.5 g l^{-1}, MgSO4 0.5g l^{-1}, NaNO3 0.5g l^{-1}, FeSO4 0.01 g l^{-1}, pH 7.4) with a 7 mm wide strip of filter paper

(FP) and one drop of 10 mM glucose in glass culture tubes, where n=3. Glucose is suggested to induce

cellulase production. The cultures were incubated for a maximum of 10 days and viewed daily for visual evidence of filter paper degradation. Those strains capable of completely degrading the filter paper within 96 h were selected for further quantitative analysis. The test was done using aerobic culture techniques and repeated under limited oxygen conditions by sealing the tubes with parafilm.

2.3.7. Total cellulase activity assay

Two isolates (E2 and E4), displaying the greatest cellulase activity qualitatively were selected for further study and quantification of total cellulase activity. Isolate E1 and E2 were grown (n=3), as similarly described in 5 mL of Dubois salts media with FP (1%, w/v) or CMC (1%, w/v) in glass culture tubes, under limited oxygen conditions at 28°C, shaking at 180 rpm. The cultures were incubated for 48 h and cellulase activities were measured. One militre of culture (n=3), was removed and centrifuged at 17,000 g for 1 min and the supernatant containing enzymes were used in the assay. A microplate-based filter paper assay using the DNS method to measure reducing sugars, modified from Xiao *et al.* 2004 [7], was used to measure the total cellulase activity for the two isolates displaying the highest cellulolytic activity. Modifications included the use of 50mM TrisHCl buffer, pH 7 in place of 50 mM NaAc buffer, pH 4.8. Bacterial enzymes do not work efficiently at such low pH. Additionally, the enzymes (20 μl) with the buffer (40 μl) and filter paper substrate were allowed to incubate at 50°C for 2 h instead of 1 h, due to the known smaller quantities of enzymes produced by bacteria. All samples were loaded to the microtitre plate in triplicate.

2.4 Results

2.4.1. Carboxymethyl cellulase activity

A total of 53 isolates were described based on size, colour, and morphology, labeled and photographed for a database (not shown here). From the database 30 of 53 isolates were removed due to similar size, colour and morphological characteristics. The resulting 23 isolates were then tested on CMC

agar for cellulase activity; 19 of the 23 isolates exhibited cellulase activity and are shown in the photographs of Figure 1 along with positive (*Cellulomonas xylanilytica*) and negative (*E. coli* JM109) controls. The following 6 cellulase-producing isolates had the greatest halos after 48h incubation on CMC agar: S1, S2, S3, S4, E2, E4 (Figure 1). The halos were measured in centimeters using a standard ruler. The diameters of the halos can be seen in Figure 2 plotted beside each genus in the phylogenetic tree.

2.4.2. 16S rDNA amplification

Genomic DNA was successfully isolated from all 19 cellulase-producing isolates using Gram positive DNA isolation methods. The universal 16S rDNA primers were used in conjunction with PCR to successfully amplify 16S rDNA gene fragments from all 19 isolates. Confirmation of the 16S rDNA gene fragments was validated by a band on a 1% agarose gel with an approximate expected size of 800 bp.

2.4.3. Sequencing and sequence analysis of 16S rDNA PCR products

Sequencing results were successfully obtained for all 19 different 16S rDNA PCR products. The resulting sequences were inputted to the nucleotide blast feature of the NCBI database to obtain possible identities based on homology. From BLAST search results, genera of all 19 isolates were determined based on 97-99% homology. The nucleotide BLAST results are shown in Table 1. The majority of sequences yielded 99% homology in nucleotide database with very few as low as 97% homology. All 19 isolates belong to genera of Gram positive bacteria, several of which were shown to belong to the genus *Bacillus* and *Paenibacillus*, while one strain from the genus *Microbacterium* and *Streptomyces* were also found (Table 1).

2.4.4. Phylogenetic analysis of 16S rDNA sequences

The sequences were then inputted into a sequence alignment program called ClustalX. An alignment was then done using UPGMA algorithm which finds the relatedness between the isolates assuming that the rate of evolution is constant. The aligned sequences were then uploaded into a program called TreeView

which allows us to view the phylogenetic tree produced from the alignment information using the UPGMA algorithm. The phylogenetic tree displays two main groups of Gram positive bacteria; the Firmicutes (blue) more closely related than the Actinobacteria (red). The Firmicutes are made up of *Bacillus* and *Paenibacillus* sp., while the Actinobacteria include the genera *Streptomyces, Microbacterium* and positive control *Celluomonas xylanilytica*. The yellow to green colour legend represents a visual of the halo diameter (cm) from smallest to greatest halo, respectively. Data was collected from the CMC plates. The *Paenibacillus* sp. all have relatively larger halos and the *Bacillus* sp. has halos of varying sizes. The Actinobateria exhibits a variety of halo sizes and *Streptomyces* seems to be the greatest producer of cellulases in this Phylum (Figure 2).

2.4.5. Filter paper activity

All of the positive cellulase-producing isolates were grown with FP as a sole carbon with one drop of 10 mM glucose to possibly induce cellulase production [8], for qualitative observation of filter paper activity. This was done in both aerobic and oxygen limited environments. The ability to degrade filter paper, more than likely represents the production of more than one type of enzyme and the ability to degrade crystalline cellulose, thereby being a more efficient cellulase-producing isolate. The following two strains: E2 and E4 were found to completely degrade the filter paper cellulose in 72 and 96 h incubation, respectively, as can be seen in Figure 3.

2.4.6. Total cellulase activity

The total cellulase activity is determined by the amount of glucose and cellobiose released from filter paper after 2 h incubation and is referred to in glucose equivalents. Similarly, the CMCase activity is also a measure of the glucose and cellobiose released however after 20 min incubation; it is also referred to as glucose equivalents. The activity for total cellulases was evaluated for whole cells of *Paenibacillus* sp. E2 and E4. It was found that using CMC as the culture carbon substrate, E2 and E4 total cellulase activity was 1587 ± 215 and 1652 ± 61 nM of glucose equivalents, respectively, not significantly different.

However, when FP was used as the cellulase inducing substrate, total cellulase activity of E2 and E4 was 202±66 and 260±157 nM of glucose equivalents. No significance difference in total cellulase activity was observed between E2 and E4; however, CMC induced greater activity. The quotient of CMC to filter paper was 8±0.4 and 6±0.1 for E2 and E4, respectively (Table 2).

2.5 Discussion

Several isolates could be recovered by aerobic spread plates from the different industrial and commercial samples. For those isolates displaying cellulase activity on the CMC containing plates four different genera of isolates including *Bacillus*, *Paenibacillus*, *Microbacterium*, and *Streptomyces* species were found. According to our phylogentic analysis, these bacteria can be grouped into two main Phyla based on sequence homology: Actinobacteria and Firmicutes. Both Phyla consist of Gram positive bacteria distinguished by high and low GC content, respectively; and both groups of bacteria contain species capable of degrading organic materials. Thus, it is not surprising that many of the genera can produce cellulases. Several strains of *Paenibacillus*, *Bacillus, Microbacterium* and *Streptomyces* have been found to produce cellulases and their cellulases have been well studied; these strains represent important cellulase degrading genera.

For example, researchers have characterized a novel endoglucanase (Cel9P) from a newly isolated *Paenibacillus* sp. BME-14. Endoglucanase Cel9P displayed 65% of its maximal activity at 5°C, which could be beneficial for some industries which have processes at lower temperatures [8]. Similarly, in other newly isolated *Paenibacillus* sp., multienzyme complexes called cellulosomes have been characterized in the degradation of lignocellulosic substrates [9-11]. Cellulosomes, such as the cellulosome of *Clostridium thermocellum* can have high efficiency for the degradation of crystalline celluloses, higher than that of *Trichoderma reesei* [12]. In addition, cellulases have also been well characterized in *Bacillus* species; most recently a unique *Bacillus* sp. was observed to maintain up to 70%

stable CMC activity at a range of pH 6-8 [13]. Similarly, many of the modular enzymes present in *Paenibacillus* are also present in *Bacillus* species [10].

Moreover, *Microbacterium* sp. displaying cellulase activities have been isolated from a variety of environmental samples and uniquely this strain has been isolated from the gut of termites [14]. One *Microbacterium* sp. exhibited particularly high filter paper activity and xylanase activity when a consortium of aerobic cellulase producing bacteria was studied [15].

Additionally, *Streptomyces* sp., have also been previously studied by researchers for cellulase production and found to produce a variety of unique cellulases including some of which were found to be thermoalkotolerant [16,17]. Also interestingly, *Streptomyces* sp. has been used in successful co-culturing trials. They have been found to work synergistically with *Thermomonospora fusca* and *Trichoderma reesei* to degrade cellulose [18].

All of the cellulase producing bacteria isolated and identified in this study have potential for further use and study, such as looking at individual enzyme activities to isolate efficient or novel cellulases with unique characteristics, or potential to use the strains to create microbial consortia with a high efficiency for degrading complex cellulose containing biomass such as lignocellulose.

Evaluating cellulase production between isolates can be a challenge because bacteria produce multiple types of cellulases (endoglucanase, exoglucanase, and β-glucosidase), which can be found to exist as free extracellular enzymes as well as found in enzyme complexes or cellulosomes expressed on the cell membrane [19]. Thus, we initially use qualitative tests such as the CMC test and filter paper degradation test. CMC agar allows us to identify isolates with cellulase activity on soluble cellulose such as CMC thus representing mainly endoglucanase and beta-glucosidase activities [5]. Secondly, we then screened isolates displaying cellulase activity on CMC for activity on crystalline insoluble cellulose such as filter paper. Due to the crystalline structure of filter paper, degradation of the filter paper would imply

multiple cellulase activities including exoglucanase activities because these enzymes work in crystalline regions [20]. From these tests we could select isolates displaying the greatest activity based on ability to degrade soluble and crystalline cellulose for quantitative analysis of FP activity. All isolates may vary in growth properties which would not allow us to easily compare and quantify cellulase activities of all cellulase positive isolates in an equal manner. Difficulty also arises because some strains may secrete enzymes to solution while others may harbor enzymes on the cell surface or internally, and still some cellulase may end up in solution from cell lysis [21]. Additionally, some bacteria grow more rapidly than others, and cellulase production may be induced by different substrates for varying species. Thus, using qualitative screening methods is essential to narrow down isolates which may be more unique for further cellulase study in the future.

Narrowing down our isolates led us to the greatest cellulase producers, *Paenibacillus* sp. E2 and *Paenibacillus* sp. E4 for further analysis. These isolates displayed some of the greatest halos on CMC agar (Figure 1) and were the only strains capable of completely degrading filter paper after 72 and 96 h incubation, respectively, under oxygen limited conditions (Figure 3); qualitatively speaking cellulase activity in these strains was greater than the positive control. The cellulase activity could be further studied under facultative anaerobic conditions. Similarly, researchers have shown that under anaerobic conditions, *Paenibacillus* species will exhibit high levels of xylanases which can degrade xylan, a more branched portion of the cell wall [11].

Focusing on *Paenibacillus sp.* E2 and E4 for further study, it was shown that after 48 h shaking incubation in oxygen limited condition with CMC and FP as the cellulase inducing substrate isolate E2 displayed total cellulase activities of 1587 ± 215 and 202 ± 66 nM of glucose equivalents, respectively. Similarly, isolate E4 displayed total cellulase activity of 1652 ± 61 and 260 ± 157 nM of glucose equivalents for CMC and FP, respectively. Results show that 48h growth with CMC can induce more cellulases than 48 h growth with FP by approximately 8.1X and 5.5X for E2 and E4, respectively. There was no

difference in total cellulase activity for either cellulosic substrate when whole cells were used versus when cells were lysed, thus data were not shown. This may suggest a high amount of cellulases are on the outside surface of the cells or secreted in the medium but not internalized.

Our results show that *Paenibacillus* species E2 and E4 may be good potential candidates for biorefining and the ultimate production of bioethanol and additional value-added bioproducts such as organic acids. These isolates are of particular interest because cellulase activities were higher and comparable to the well-known positive control, *Cellulomonas xylanilytica*. Future work will be done on E2 and E4 to optimize cellulase production and evaluate individual cellulase activities to look for novel cellulases.

According to this study, our isolation, screening and identification methods were quick and efficient for allowing us to identify several good cellulase producing bacteria from a wide variety of samples. Moreover, we were able to distinguish the isolates displaying the greatest cellulase activity for future study. Finding naturally occurring cellulase degrading bacteria from the environment is important in the field of biorefining to help overcome costly hurdles in the biorefining process. All of our cellulase positive isolates may be an integral part of future work to develop good cellulases or produce efficient cellulase producing systems such as microbial consortia which can be used for industry. Isolation and characterization may provide a good starting point for the discovery of such beneficial enzymes.

References

1. Schneider SH. The greenhouse effect: science and policy. Science 1989;243:771-781.

2. Greene N. Growing energy. How biofuels can help end America"s oil dependence. Nat Res Def Council Rep 2004:1-86.

3. Davies G, Henrissat B. Structures and mechanisms of glycosyl hydrolases. Structure 1995;3:853-859.

4. Lamed R, Setter E, Kenig R, Bayer EA. The cellulosome: A discrete cell surface organelle of clostridium thermocellum which exhibits separate antigenic, cellulose-binding and various cellulolytic activities. Biotechnol Bioeng 1983;13:163-181.

5. Kasana RC, Salwan R, Dhar H, Dutt S, Gulati A. A rapid and easy method for the detection of microbial cellulases on agar plates using gram's iodine. Curr Microbiol 2008;57:503-507.

6. Giannino ML, Aliprandi M, Feligini M, Vanoni L, Brasca M, Fracchetti F. A DNA array based assay for the characterization of microbial community in raw milk. J Microbiol Methods 2009;78:181-188.

7. Xiao Z, Storms R, Tsang A. Microplate-based filter paper assay to measure total cellulase activity. Biotechnol Bioeng 2004;88:832-837.

8. Fu X, Liu P, Lin L, Hong Y, Huang X, Meng X, Liu Z. A novel endoglucanase (Cel9P) from a marine bacterium Paenibacillus sp. BME-14. Appl Biochem Biotechnol 2010;160:1627-1636.

9. Waeonukul R, Kyu KL, Sakka K, Ratanakhanokchai K. Isolation and characterization of a multienzyme complex (cellulosome) of the Paenibacillus curdlanolyticus B-6 grown on Avicel under aerobic conditions. J Biosci Bioeng 2009;107:610-614.

10. Pastor FI, Pujol X, Blanco A, Vidal T, Torres AL, Diaz P. Molecular cloning and characterization of a multidomain endoglucanase from Paenibacillus sp BP-23: evaluation of its performance in pulp refining. Appl Microbiol Biotechnol 2001;55:61-68.

11. Pason P, Kosugi A, Waeonukul R, Tachaapaikoon C, Ratanakhanokchai K, Arai T, Murata Y, Nakajima J, Mori Y. Purification and characterization of a multienzyme complex produced by Paenibacillus curdlanolyticus B-6. Appl Microbiol Biotechnol 2010;85:573-580.

12. Ng TK, Zeikus JG. Comparison of Extracellular Cellulase Activities of Clostridium thermocellum LQRI and Trichoderma reesei QM9414. Appl Environ Microbiol 1981;42:231-240.

13. Yang D, Weng H, Wang M, Xu W, Li Y, Yang H. Cloning and expression of a novel thermostable cellulase from newly isolated Bacillus subtilis strain I15. Mol Biol Rep 2010;37:1923-1929.

14. Wenzel M, Schonig I, Berchtold M, Kampfer P, Konig H. Aerobic and facultatively anaerobic cellulolytic bacteria from the gut of the termite Zootermopsis angusticollis. J Appl Microbiol 2002;92:32-40.

15. Okeke BC, Lu J. Characterization of a defined cellulolytic and xylanolytic bacterial consortium for bioprocessing of cellulose and hemicelluloses. Appl Biochem Biotechnol 2010;163:869-881.

16. George J, Arunachalam R, Paulkumar K, Wesely EG, Shiburaj S, Annadurai G. Characterization and phylogenetic analysis of cellulase producing Streptomyces noboritoensis SPKC1. Interdiscip Sci 2010;2:205-212.

17. Alani F, Anderson WA, Moo-Young M. New isolate of Streptomyces sp. with novel thermoalkalotolerant cellulases. Biotechnol Lett 2008;30:123-126.

18. Jung ED, Lao G, Irwin D, Barr BK, Benjamin A, Wilson DB. DNA sequences and expression in Streptomyces lividans of an exoglucanase gene and an endoglucanase gene from Thermomonospora fusca. Appl Environ Microbiol 1993;59:3032-3043.

19. Maki M, Leung KT, Qin W. The prospects of cellulase-producing bacteria for the bioconversion of lignocellulosic biomass. Int J Biol Sci 2009;5:500-516.

20. Dashtban M, Maki M, Leung KT, Mao C, Qin W. Cellulase activities in biomass conversion: measurement methods and comparison. Crit Rev Biotechnol 2010;30:302-309.

21. Berg B. Cellulase location in Cellvibrio fulvus. Can J Microbiol 1975;21:51-57.

Table 1. The BLAST search results for the sequenced cellulase-producing isolates.

Isolate	Homology (%)	Genus
B1	99	*Microbacterium*
B2	99	*Bacillus*
B3	99	*Bacillus*
B4	99	*Bacillus*
B5	98	*Bacillus*
B6	99	*Bacillus*
B7	99	*Streptomyces*
E1	99	*Bacillus*
E2	98	*Paenibacillus*
E3	99	*Paenibacillus*
E4	98	*Paenibacillus*
SM1	97	*Bacillus*
SM2	98	*Bacillus*
SM3	98	*Bacillus*
SM4	99	*Paenibacillus*
SM5	97	*Bacillus*
WC1	99	*Bacillus*
WC2	98	*Bacillus*

Table 2. Total cellulase activity of *Paenibacillus* sp. E2 and E4 shown in glucose equivalents (nM) after 48 h growth with CMC (1%,w/v).

Substrate (1%, w/v)	Total Cellulase Activity (glucose equivalents (nM))	
	Paenibacillus E2	*Paenibacillus* E4
Carboxymethyl cellulose	1652 ±61	1456±30
Filter paper	202±66	260±157
Quotient (CMC/FP)	8±0.4	6±0.1

Figure Legend

Figure 1. Nineteen cellulase-producing isolates grown on CMC for 48 h at 28°C, grouped based on the samples they were derived from, and a positive and negative control, *C. xylanilytica* and *E. coli* JM109, respectively.

Figure 2. Phylogenetic tree produced from the alignments of 16S rDNA fragments from the isolates, presented in TreeView. Closer related isolates outlined in blue belong to the phylum Firmicutes and the most distantly related isolates outlined in red belong to the phylum Actinobacteria. The diameter of halos the isolates produced on CMC agar is respectively shown with a colour scale indicating small to large halos, qualitative cellulase activity.

Figure 3. Qualitative results for the 2 isolates capable of completely degrading filter paper within 96 h incubation: A) Negative control (*E. coli* JM109), B) Positive control (*C. xylanilytica*), C) *Paenibacillus* E2 and D) *Paenibacillus* E4.
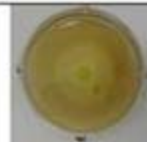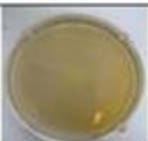
Figure 1

Figure 2

Figure 3

Chapter 3: Newly isolated and characterized bacteria with great application potential for decomposition of lignocellulosic biomass

Authors: Miranda L Maki, Amna Idrees, Kam Tin Leung, Wensheng Qin

3.1 Abstract

This study focuses on the isolation and characterization of bacteria from municipal waste and peat to determine those with good potential for modification and decomposition of lignocellulosic biomass for industrial application. Twenty cellulase-producing bacteria belonging to four major phyla: Firmicutes, Actinobacteria, Proteobacteria and Bacteriodetes were found when screened on CMC-containing agar. Six isolates also exhibited activities towards filter paper as the sole carbon source in salts media; while 12 exhibited activities towards xylan when screened on xylan-containing plates. Moreover, five isolates survived in and increased absorbance of 1% black liquor in salts media by an average of 2.07-fold after 21 days incubation. Similarly, these five isolates increased the absorbance of 0.1% pure lignin at 280 nm in salts media, indicating modification of lignin. Additionally, FTIR analysis of 1% barely straw treated for 21 days with these 5 strains showed a preference for consumption of hemicelluloses over lignin; however a change in lignin was observed. A *Bacillus* strain (55S5) and *Pseudomonas* strain (AS1), respectively, displayed the greatest potential for lignocellulose decomposition due to a variety of cellulase activities, as well as xylanase activity, and modification of lignin. Several of these isolates have good potential for industrial use in the degradation of lignocellulosic biomass.

***Keywords:** Biodegradation, lignocellulase-producing bacteria, xylanase, lignase, Bacillus, Pseudomonas*

3.2 Introduction

In recent years, global climate change and rising fuel costs have caused an increased awareness and potential for renewable fuels sources such as biofuels from lignocellulosic biomass [Schneider, 1989]. There are many reports of microorganisms such as bacteria and fungi which possess hydrolytic activities on cellulose, the major component of lignocellulosic biomass. However, there are several limitations to this approach; for example, lignocellulosic biomass is also comprised of some hemicelluloses (heterologous polymers of 5- and 6-carbon sugars), the majority of which have a xylan backbone [Scheller and Ulvskov, 2010]. Furthermore, celluloses and hemicelluloses are entrapped by lignin, a more complex aromatic polymer. The combination of mainly these components make up the structure of plant cell walls and provide the plants with structural integrity and protect plants from such things as disease, pests, wind and mechanical wounds.

Lignocellulosic biomasses such as agricultural residues and energy crops currently undergo extensive pretreatment using acid hydrolysis and steam or high temperature treatments to remove lignin and hemicellulose to expose the cellulose for enzymatic hydrolysis [Galbe and Zacchi, 2007]. Thus, microorganisms with abilities to decompose or modify lignin and hemicellulose in addition to cellulose have greater potential in industrial production of biofuels, as they can help reduce the associative costs with pretreatment.

Researchers have typically focused on one group of enzymes during isolation, such as cellulases, hemicellulases or lignases. For example, white rot fungi are best studied for their remarkable ability to degrade lignin [Otjen and Blanchette, 1982]. However, anaerobic bacterium *Clostridium thermocellum* and aerobic fungi *Trichoderma reesei* have been well studied for their abilities to efficiently degrade crystalline cellulose [Ng and Zeikus, 1981]. Nonetheless, none of these microorganisms is efficient at cellulolytic, hemicellulolytic and ligninolytic activities simultaneously, rendering the opportunity for discovery of better lignocellulase-producing isolates. Here we define "lignocellulase-producing" as

microorganisms which can produce different enzymes such as cellulases, hemicellulases and lignases collectively, for the decomposition of lignocellulosic biomass.

We have focused on the isolation of lignocellulolytic bacteria. That is, bacteria which produce a greater variety of enzymes including here cellulases, xylanases and lignases in hopes of finding bacteria which can have an overall greater decomposing impact on complex biomass which could thus be potentially applied in industrial practices. Use of such strains in industry could thereby reduce the need for extensive pretreatments or reduce limitation on the types of biomass used for cost-competitive markets.

Fungi and yeasts have frequently been applied in the development of industrial enzymes. However, bacteria have several advantages over the use of such microorganisms, such as many strains have short generation times and can be easily cultured making the use of bacteria in the biofuel industry more amenable. Additionally, bacteria also have increased resilience to environmental stresses due to their biochemical versatility (i.e. temperature variations, salinity, oxygen limitation and change in pH) [Daniel and Nilsson, 1998].

Several studies have focused time and again on isolation strategies for targeting bacteria with more specific activities such as efficient cellulase producing bacteria, yet some studies isolated bacteria with cellulase and xylanase activity, however very few with lignase activities in addition to the previous [Maki et al., 2009; Maki et al., 2011; Sizoza et al., 2011]. In this study, we developed a strategy for finding bacteria which can produce celllulaes, xylanases and potential lignases, and therefore can be more efficient in decomposing lignocellulosic biomass making them more practical in industrial use.

In this study, several efficient aerobic cellulase-producing microorganisms were isolated from various sites within a municipal landfill and peat core samples from a poor nutrient fen. The purpose was to characterize lignocellulosic abilities of all cellulase positive isolates and identify those isolates displaying the greatest variety of activity towards lignocelluolosic biomass for the possible more practical use in large scale biorefining.

3.3 Materials and Methods

3.3.1. Lignocellulosic samples and media

The lignocellulase-producing bacteria were isolated from several samples of two main sources: municipal waste and peat. The first set of samples was obtained from the City of Thunder Bay Solid Waste and Recycling Facility, Ontario, Canada. The second set of samples was obtained using a peat corer to 1.5 m deep of a poor fen near Raith, Ontario, Canada.

The growth media used in the experiments include R2A agar (0.5 g/l yeast extract, 0.5 g/l protease peptone, 0.5 g/l casamino acids, 0.5 g/l glucose, 0.5 g/l soluble starch, 0.3 g/l dipotassium phosphate, 0.5 g/l magnesium sulfate $7H_2O$, 0.3 g/l sodium pyruvate, 15.0 g/l agar), LB liquid media (10.0 g/l peptone, 5.0 g/l yeast extract, 5.0 g/l NaCl), and carboxymethyl cellulose agar (0.5 g CMC, 0.1 g $NaNO_3$, 0.1 g K_2HPO_4, 0.1 g KCl, 0.05 g $MgSO_4$, 0.05 g yeast extract, 1.5 g agar, per 100 ml ddH_2O, pH 7.4) [Kasana et al., 2008].

3.3.2. Isolation of bacteria from lignocellulosic samples

The method of Maki *et al.*, as utilized in the previous chapter was used to isolate bacteria. Briefly, 1 g of each sample was suspended in 20 ml of sterile potassium phosphate buffer solution (1X PPB) by vortexing for 2 min on maximum speed. Following, a 100X serial dilution of the suspension was made in PBS. Thereafter, 200 µl of each dilution in the series was spread onto the surface of R2A agar using the standard spread plate technique. All plates were incubated at 28°C for 24 h before isolating individual colonies and then they were incubated for an additional 48 and 72 h to allow growth of slower growing microorganisms. From the growth observed over 24, 48 and 72 h, various colonies were selected based on their morphology, size and colour. The colonies selected were then streaked out on separate R2A plates until purity. After purification, the cultures were compared visually to eliminate those of similar size, morphology and colour [Maki et al., 2011].

3.3.3. Screening for carboxymethyl cellulase activity

The method described by Maki *et al.* in 2011, was used to screen isolates for carboxymethyl activity. As described, isolates were grown in 10 ml of LB broth for 24 h, at 28°C, shaking at 200 rpm, slower growing isolates were left to incubate for an additional 48 h. The positive control used was *Cellulomonas xylanilytica* XIL11 [Rivas et al., 2004]. This strain was also grown in the LB medium; however it required incubation for a 5 day period using the same growth conditions. *C. xylanilytica* requires 4 days growth on solid media to detect colonies, it was grown an additional day to allow 48 h with visible colony. The negative control used was *Escherichia coli* JM109, also grown in LB broth overnight; however, it grows at 37°C for 18 h. All resulting broth cultures (isolates, positive control and negative control) were tested for cellulase activity via the Gram"s iodine method [Kasana et al., 2008]. Briefly, 5µl of each broth culture were singly dropped onto a plastic Petri dish containing carboxymethyl cellulose (CMC) agar, incubated for 48 hours at 28°C and flooded with Grams iodine solution (2.0 g KI and 1.0 g I, per 300 ml ddH$_2$O) for 5 minutes to visualize and photograph the cellulase activity. The agar containing CMC stains brown and areas without CMC are clear, described here as halos, as seen in Figure 1. Halo diameters were measured using a ruler for a semi-qualitative comparison of cellulase activity among the isolates after 48 h incubation. The halo measurement is used to relate cellulase activity to bacteria position on the phylogenetic tree as shown in Figure 2, as similarly done by Maki *et al.* [2011].

3.3.4. DNA isolation and 16S rDNA amplification

The cellulase producing isolates as well as the positive control were grown up in LB broth for 24 h at 28°C. DNA was isolated from each isolate broth culture using the Fungi/Yeast Genomic DNA Isolation Kit from Norgen Biotek Corporation, Canada. The resulting isolated DNA was used as a template in a PCR reaction to amplify a region of the 16S rRNA gene. Universal primers designed within conserved regions of the 16S rRNA gene for Eubacteria were used: HAD-1 (5´-GACTCCTACGGGAGGCAGCAGT) and E1115R (5´-AGGGTTGCGCTCGTTGCGGG), they amplify

approximately a 796 bp fragment [Giannnino et al. 2009]. The PCR reaction mixtures contained 10 ng of genomic DNA individually from each positive isolate, 10 pmol of both forward and reverse primers, 10x Taq buffer with KCl, 25 mmol/l $MgCl_2$, 0.2 mmol deoxynucleoside triphosphate, and 5 U Taq DNA polymerase per 50 μl reaction. The PCR program was as follows: primary denaturation 3 minutes at 95°C, followed by 33 amplification cycles consisting of denaturing at 95°C for 1 minute, annealing for 1 minute at 63°C, and extension at 72°C for 1 minute, upon completion of 33 amplification cycles a final extension step was done at 72°C for 10 minutes. The PCR products were then viewed on a 1% agarose gel to confirm size, quantity and purity. Then, PCR products were sequenced using standard run modules on the ABI 3730xl automatic sequencer (Eurofins MWG Operon, Canada).

3.3.5. Isolate Identification and Relatedness

Sequencing results were individually inputted online into the nucleotide blast tool through the NCBI database (http://blast.ncbi.nlm.nih.gov/) to identify the possible genera of the isolates. Sequencing results of the isolates and positive control were also inputted into a sequence alignment program called ClustalX to determine the phylogenetic relatedness of the different species. They were aligned using the UPGMA algorithm, which considers the rate of evolution to be constant between species, to develop a phylogenetic tree based on sequence homology. The resulting alignment was opened into a program called TreeView which allowed the phylogenetic tree to be viewed.

3.3.6. Filter paper activity

Isolates displaying cellulase activity on the CMC plates were further screened for quality of cellulase activity by transferring 100 μl of an overnight culture to 5 ml of Dubois salts media (K2PO4 1 g/l, KCl 0.5 g/l, MgSO4 0.5 g/l, NaNO3 0.5 g/l, FeSO4 0.01 g/l, pH 7.4) with a 7 mm wide strip of filter paper (FP) and one drop of 10 mM glucose in glass culture tubes, in triplicate (n=3). The cultures were incubated for a maximum of 10 days at 28°C, shaking at 180 rpm and viewed daily for visual evidence of filter paper degradation. The release of reducing sugars by those strains capable of completely degrading

the filter paper within 96 h was measured and all triplicates using the DNS method [Xiao et al., 2004] and was expressed as nM glucose equivalents.

3.3.7. Screening for xylanase activity

Qualitative evidence for xylanase activities of all of the cellulase-positive isolates was evaluated using the same method described for the screening of cellulase activity. However, for the xylanase activity, 0.5 g of Beechwood xylan (Sigma Aldrich) was substituted for CMC. Once again, the presence of halos after Gram¨s staining in room temperature after 48 h incubation at 28°C, indicated evidence of xylanase activities.

3.3.8. Modification of black liquor and cell survival

Overnight cultures isolate were prepared by inoculating one colony to 10 ml of LB and incubated at 28°C, shaking 180 rpm. From an overnight culture 200 μl of cells for each isolate were inoculated in triplicate to 100 ml of Dubois salts media supplemented with 1% (w/v) black liquor from Resolute Pulp and Paper, ON (pH 6.5) and incubated at 30°C, shaking 150 rpm. Samples were collected at days 0, 1, 3, 5, 7, 10, 14 and 21 (n=3). For decolorization experiments, 500 μl of cell suspensions and one untreated sample were diluted with 500 μl of PPB then centrifuged at 17,000 g in a microcentrifuge tube for 4 min. In triplicate, 300 μl of the supernatant were loaded into a microtitre plate and the absorbance or intensity of colour (at 425 nm), of the samples were measured by an xMark Microplate Spectrophotometer (Bio-Rad, Canada). Simultaneously, to determine cell survival, 500 μl of cell samples were collected for the drop plate counting technique on LB agar to determine cell density (CFU/ml). Additionally, a change in pH of black liquor after treatment with the isolates was measured using an Accument Combination pH electrode with silver reference (Thermo Fisher Scientific, Canada).

3.3.9. Reflectance of lignin at 280 nm

Isolates capable of modifying the colour of black liquor at 425 nm were selected for further analysis on 98% pure lignin extracted from black liquor. For each, 100 µl of an overnight culture were inoculated in triplicate to 6 ml of Dubois Salts media supplemented with 0.1% (w/v) pure lignin, 0.3% (w/v) peptone and 0.5% (w/v) glucose, and incubated at 30°C, 150 rpm for 21 days. All tubes were sealed with parafilm to eliminate evaporation. After 21 days incubation, 1 ml aliquots of each cell suspension and one untreated sample, were centrifuged for 4 min at 17,000 g. Following, supernatant was diluted 1000x in 1X PPB before reading the reflectance at 280 nm. The data is presented in Figure 6 as percentage change in lignin degradation which was calculated by substracting the absorbance of the untreated sample from the absorbances of the isolates and dividing the product by the absorbance of the untreated.

3.3.10. FTIR analysis of isolates on barely straw

The isolates displaying modification of lignin were further analyzed for lignase activities using FTIR analysis after 21 days incubation with 1% (w/v) barely straw in Dubois Salts media supplemented with 0.3% (w/v) peptone and 0.5% (w/v) glucose. In triplicate, 1 ml of isolate overnight cultures were inoculated to 100 ml of barley straw media and incubated at 30°C, shaking at 150 rpm (n=3). After 21 days incubation, isolate cultures and one untreated barely straw control were filtered through Whatman filter paper and washed once with 10 ml of distilled water. The barely straw was collected and oven dried at 60°C for 48 h prior to FTIR spectra analysis. Dried treated and untreated barely straw samples were loaded in triplicate directly to a Bruker Tensor 37 Fourier Transform Infrared Spectrophotometer equipped with an InGaAs detector (Bruker Optics Ltd., Canada). Peak height and area were measured by constructing a baseline connecting the lowest data points on either side of the peak using an interpolation calculation through Excel software. Areas were then used to compare to control and determine percentage preference [Pandey and Pitman, 2003].

3.4 Results

3.4.1. Carboxymethyl cellulase activity

A total of 57 isolates were described based on size, colour, and morphology, labeled and photographed for a database (not shown here). From the database, 25 of 57 isolates were removed due to similar size, colour and morphological characteristics. The resulting 32 isolates were then tested on CMC agar for cellulase activity; 20 of the 32 isolates exhibited cellulase activity and are shown in the photographs of Figure 1 along with positive (*Cellulomonas xylanilytica*) and negative (*E. coli* JM109) controls. The following 7 cellulase-producing isolates had the greatest halo diameters after 48h incubation on CMC agar: CDS1B, CDS2B, AS2B, CTS1A, CTS2, GH2OS1 and 6S4 (Figure 1). The diameters of the halos were measured in centimeters using a standard ruler. The diameters of the halos can be seen in Figure 2 plotted beside each genus in the phylogenetic tree.

3.4.2. 16S rDNA amplification

Genomic DNA was isolated from all 20 cellulase-producing isolates using Gram positive DNA isolation methods. The universal 16S rRNA gene primers were used in conjunction with PCR to amplify 16S rRNA gene fragments from all 20 isolates. Confirmation of the 16S rRNA gene fragments was validated by a band on a 1% agarose gel with an approximate expected size of 800 bp.

3.4.3. Sequencing and sequence analysis of 16S rDNA PCR products

Sequencing results were obtained for all 20 different 16S rRNA gene PCR products. The resulting sequences were inputted to the nucleotide blast feature of the NCBI database to obtain possible identities based on sequence similarity. The genera of all 20 isolates were determined from the BLAST search, based on a high sequence similarity ranging from 96-100%. The nucleotide BLAST results are shown in Table 1. The majority of sequences yielded 100% similarity in nucleotide database with very few as low as 96 and 97% sequence similarity. Many of the 20 isolates belong to genera of Gram positive bacteria,

such as the genus *Bacillus, Paenibacillus*, *Rhodococcus*, *Arthrobacter*, *Exiguobacterium* and *Microbacterium*. Some strains of Gram negative bacteria were also found belonging to the genera *Pseudomonas*, *Aeromonas, Duganella* and *Chryseobacterium* (Table 1).

3.4.4. Phylogenetic analysis of 16S rDNA sequences

The phylogenetic tree displays two main groups of more closely related Gram positive bacteria; the Firmicutes and the Actinobacteria, while the more distantly related Gram negative bacteria were grouped into two main phyla, the Proteobacteria and Bacteriodetes. *Chryseobacterium* the only genera belonging to the phylum Bacteriodetes was least related to all of the isolates. The yellow to green colour legend represents a visual of the halo diameter (cm) from smallest to greatest halo on CMC agar, respectively. In this study, isolates belonging to the phylum Firmicute and Proteobacteria contain genera of bacteria with relatively larger halos than genus of bacteria found belonging to the phyla Actinobacteria and Bacteriodetes (Figure 2).

3.4.5. Filter paper activity

All of the positive cellulase-producing isolates were grown with FP as a sole carbon with one drop of 10 mM glucose to possibly induce cellulase production, for qualitative observation of filter paper activity. This was done in both aerobic and oxygen limited environments. The ability to degrade filter paper, more than likely represents the production of more than one type of enzyme and the ability to degrade crystalline cellulose. The following 6 strains: 6S1, 55S5, AS1, CDS1B, CH2OS1, and CTS1B were found to completely degrade the filter paper cellulose within 96 h incubation similar to the positive control, as can be seen in Figure 3. Degradation was confirmed by the amount of sugars released into solution after complete degradation for *Bacillus* sp. 6S1, *Bacillus* sp. 55S5, *C. xylanilytica* (+), *Pseudomonas* sp. AS1, *Aeromonas* sp. CDS1B, *Bacillus* sp. CH20S1, *Bacillus* sp. CTS1B and found to be similar at 782.6, 978.0, 1043.5, 847.8, 913.0, 608.7, and 1065 nmoles of glucose equivalents, respectively. There were no sugars detected in the negative control (*E. coli*).

3.4.6. Xylanase activity

The 20 cellulase positive isolates were further screened for evidence of hemicellulase activity on xylan containing media. It was observed that 12 of 20 isolates were able to utilize xylan as a source of carbon by producing halos after staining. Isolates: *Bacillus* sp. 55S5, *Bacillus* sp. 6S1, *Bacillus* sp. CDS2B, *Pseudomonas* sp. CDS3, *Pseudomonas* sp. GH2OS1 produced the greatest halo diameters suggesting high production of xylanase, as can be seen in Figure 4.

3.4.7. Modification of black liquor and cell survival

From 20 isolates screened for growth and survival in 1% (w/v) black liquor, a total of 11 isolates were capable of surviving 21 days incubation. Absorbance at 425 nm allowed observation of change in colour of the black liquor. No isolates were capable of decreasing the absorbance after 21 days. However, 5 strains: *Pseudomonas* sp. AS1, *Microbacterium* sp. AS4, *Bacillus* sp. 65S3, *Paenibacillus* sp. 65S5 and *Bacillus* sp. CH2OS1 were capable of increasing the absorbance of black liquor after 21 days incubation of an average 10,000-fold greater than the absorbance at day 0, indicating a modification of black liquor. The pH was recorded after 21 days and for the 5 previously mentioned strains an increase in colour was related an increase in pH from 6.5 to 9.5 (Figure 5A). Similarly, these five strains could also grow and proliferate, increasing their initial cell densities on average by 50- fold and maintaining these cell densities up to 21 days incubation, as determined by cell survival in black liquor (Figure 5B).

3.4.8. Reflectance of lignin at 280nm

Modification of 0.1% (w/v) pure lignin extracted from black liquor, was observed by measuring the absorbance at 280 nm after 21 days incubation for each strain displaying modification of black liquor: AS1, AS4, 65S3, 65S3 and CH2OS1 (Figure 6). All 5 strains increased the absorbance of lignin at 280nm compared to the untreated control sample. Isolates AS1 and AS4 were capable of increasing absorbance the greatest by 23.8% and 21.8%, respectively, compared to the control. Whereas, isolate CH2OS1 only

increased absorbance by 6.3% compared to control. The remaining 2 isolates, 65S5 and 65S3 increased absorbance by 13.9 and 11.1% compared to the control, respectively.

3.4.9. FTIR analysis of isolates on barely straw

The FTIR spectra data was used to analyze the preference of all 5 previously mentioned potential lignin modifying isolates (AS1, AS4, 65S3, 65S5 and CH2O) for hemicellulase and lignin compared to cellulose, as shown in Figure 7. For comparison, the cellulase positive control (*C. xylanilytica*) which could not survive in black liquor and displayed minimal hemicellulase activity was also used. The results showed that *C. xylanilytica* had no preference for lignin and a 43.2% preference for hemicellulose compared to cellulose, as was expected. Also, displaying relatively low preferences for lignin were isolates CH2OS1 and 65S5, with 6.5 and 27.2% preferences, respectively; whereas, they preferentially consumed hemicellulose with preferences of 93.2 and 92.7%, respectively. In contrast, strains AS1, 65S3 and AS4 had greater preferences for lignin of 56.5, 49.0 and 42.0%, respectively. Additionally, they consumed hemicellulose with greater preferences of 97.6, 68.7 and 61.8%, respectively.

3.5 Discussion

A variety of bacterial isolates were recovered from different samples of municipal waste and peat. Phylogenetic analysis of 16S rRNA gene sequences of all bacteria displaying cellulase activity on CMC plates revealed four main phyla of bacteria: Actinobacteria, Firmicutes, Proteobacteria and Bacteroidetes. Within these four phyla various genera of bacteria were isolated including *Bacillus*, *Paenibacillus*, *Exiguobacterium*, *Rhodococcus*, *Arthrobacter*, *Microbacterium*, *Pseudomonas*, *Aeromonas*, *Duganella* and *Chryseobacterium*. There is little research done on cellulase, let alone lignocellulase production from genera such as *Duganella* and *Chryseobacterium*; however, it is not surprising that several of these bacteria produce cellulases as these four phyla contain important genera of bacteria capable of biodegradation of organic compounds and these species can be found ubiquitously in the environment.

Therefore, these strains represent good potential candidates for greater lignocellulolytic activities including degradation of crystalline cellulose, xylanase activities and abilities to modify or even degrade lignin.

Generally speaking, all of the isolates displayed good industrial potential for degradation of lignocellulosic biomass; however, some displayed greater potential. For example, from 20 isolated bacteria, a total of 6 could degrade filter paper in addition to soluble cellulose. Moreover, 12 isolates could degrade xylan, 5 of which were among those capable of degrading filter paper. Additionally, 5 isolates displayed modification of lignin, while 2 of these isolates: 55S5 and AS1 a *Bacillus* sp. and *Pseudomonas* sp., respectively, displayed all 3 activities including degradation of crystalline cellulose, xylanase activity and modification of lignin. In the near future, our lab will focus particular attention on isolates 55S5 and AS1 for optimization of lignocellulolytic abilities of these strains on complex biomass such as barley straw and paper mill sludge. These two isolates displayed the greatest variety of activities and have great potential for industrial applications in the degradation of more complex biomass such as agricultural residues and energy crops.

Characterization of cellulase and xylanase producing bacteria has gained an immense amount of attention due to the readily available abundance of cellulosic and hemicellulosic carbon sources in the world, which can be degraded into reducing sugars and ultimately fermented to value-added by-products such as bioethanol [Ragauskas et al., 2006]. Therefore, evaluating these activities in our isolates is pertinent to finding an efficient lignocellulosic bacterium. As a result, it was important for us to distinguish those strains which can degrade amorphous and crystalline cellulose in addition to xylanase activity. Hence, we could obtain 5 isolates displaying activities towards soluble and crystalline cellulose in addition to activities on beechwood xylan ultimately leading us to distinguish the greatest lignocellulase-producing isolates: 55S5 and AS1.

Furthermore, it is no surprise that 5 of the isolates (including 55S5 and AS1) displayed evidence for modification of lignin in black liquor and pure lignin extracted from black liquor. Several researchers reported strains of *Bacillus* and *Paenibacillus* which displayed the abilities to decolorize kraft black liquor [Chandra et al., 2007; Chandra et al., 2008 and Hassan and Amr, 2009]. Moreover, Raj in 2010, reported the decolorization of black liquor by a newly isolated bacterium, *Aneurinibacillus aneurinilyticus*. Remarkably, after 6 days incubation, *A. aneurinilyticus* was able to reduce the colour of black liquor by 58% and reduce lignin content by 43% [Raj et al., 2010]. Similarly, Chandra and Abhisshek recently reported in 2011 that mixed cultures of *Citrobacter* sp. could increase reduction of colour in black liquor to 79% and reduction in lignin to 60% after just 6 days incubation. Perhaps mixed cultures could serve to improve the activities of our isolates collective effects on lignin modification and carbohydrate degradation lending to greater lignocellulosic abilities. Most of our isolates were derived from similar sources, suggesting they co-exist in the environment and may therefore have great potential in the production of a lingocelluose-degrading bacterial consortium, which may be examined in the future.

Although members of *Pseudomonas* species have not been as readily reported in the decolorization of black liquor, they are suggested to have ligninolytic activities. For example, some *Pseudomonas* sp. have been recently described for the abilities to degrade dyes such as Malachite Green (MG) and Direct Orange 39 (Orange TGLL) with such lignases as peroxidases [Du et al., 2011 and Jadhav et al., 2010], thus explaining why our *Pseudomonas* sp. also has good potential for industrial degradation of lignocellulosic biomass.

Of 20 isolates, not all could survive and proliferate in 1% black liquor, due to the toxicity associated with lignins and modified kraft lignins, including the remaining components of black liquor. The 10 strains could survive most likely due to an activation of their stress survival response systems [Guiliordori et al., 2007], as can be seen by the initial decline and then rebound in cell density prior to 5 days incubation. Although 10 strains could adapt and survive 21 days incubation in the presence of black

liquor only 5 strains caused a change in the colour of black liquor, however the colour did not decrease in absorbance but increased, corresponding to an increase in pH. There is minimal discussion regarding such an increase in colour because decreasing the colour in black liquor is important for paper mill industries which wish to detoxify the black liquor before release to the environment.

Additionally, for the same strains, there was also an increase in the absorbance of lignin at 280 nm, while the FTIR analysis revealed that there was a greater preference for carbohydrates like hemicellulose in addition to a notable change in lignin compared to our cellulase positive control *C. xylanilytica* which could not use lignin evident by the negative value. With the support of our FTIR analysis, we suggest that strains: AS1, AS4, 65S3, 65S5 and CH2OS1 all have the ability to modify lignin however the exact mechanism is still unknown despite recent tests for lignase activities including: manganese peroxidase (MnP), lipase (LiP), and Laccase (Lac) (data not shown).

There are some speculations for the resultant increase in colour of black liquor after treatment however. Increase in reflectance at 280 nm could also be caused by increased concentration of proteins in solution which also absorb in this range as displayed in the Bradford assay [Bradford, 1976]. Moreover, some researchers have reported from ultraviolet spectra an increase in absorbance at 260 nm of white-rot fungi treated lignin; while others have reported an increase in peak absorbance to 360 nm during ultraviolet spectra analysis of *Polyporus versicolor* treated fungi. They propose the increase in absorbance to be due to structural changes such as a possible increase in acidic moieties and an increase in benzyl carbonyl groups and pheonolic units, respectively [Kirk and Lundquist, 1970; Thivend and Lebrevon, 1969]. It has also been said that the structure, including intermonomer linkages and various functional groups give microorganisms the opportunity to make limited changes without necessarily affecting significant decomposition [Kirk, 1971]. Structural changes or modifications and even possibly by-products in solution could be capable of increasing the pH which would thereby suggest the lignin is more

soluble. This could be valuable information in building a unique bacterial system for the decomposition of lignocellulosic biomass.

According to this study, we have identified two isolates: a *Pseudomonas* sp. AS1 and *Bacillus* sp. 55S5, with potential for industrial use in the conversion of lignocellulosic biomass for the production of bioethanol and other valuable by-products such as organic acids. In addition, many of our isolates characterized here also have potential in industrial use, some of which are more efficient in cellulase, and xylanase production. Others may possess undiscovered lignase genes. These isolates lay the foundation for the current exploitation of these enzymes by further investigation. Also, these strains may have great potential for developing bacterial consortia in the near future to enhance the decomposition of lignocellulosic biomass and help overcome costly hurdles being faced in the industrial production of biofuels.

Acknowledgements

References

Bradford M: A Rapid and Sensitive Method for the Quantitation of Microgram Quantities of Protein Utilizing the Principle of Protein-Dye Binding. Anal Biochem 1976; 72:248-254.

Chandra R, Raj A, Purohit HJ, Kapley A: Characterisation and optimisation of three potential aerobic bacterial strains for kraft lignin degradation from pulp paper waste. Chemosphere 2007; 67:839-846.

Chandra R, Singh S, Reddy MMK, Patel DK, Purohit HJ, Kapley A: Isolation and characterization of bacterial strains *Paenibacillus* sp. and *Bacillus* sp. for kraft lignin decolorization from pulp paper mill waste. J Gen Appl Microbiol 2008; 54;399-407.

Chandra R, Abhishek A: Bacterial decolorization of black liquor in axenic and mixed condition and characterization of metabolites. Biodegradation 2011; 22:603-611.

Daniel G, Nilsson T: Developments in the study of soft rot and bacterial decay. In: Bruce A, Palfreyman, JW (Eds.), Forest Products Biotechnology. Taylor & Francis, London, UK, 1998, pp 37–62.

Du L-N, Wang S, Li G, Wang B, Jia X-M, Zhao Y-H, Chen Y-L: Biodegradation of malachite green by Pseudomonas sp. strain DY1 under aerobic condition: characteristics, degradation products, enzyme analysis and phytotoxicity. Ecotoxicology 2011; 20:438-446.

Galbe M, Zacchi G: Pretreatment of Lignocellulosic Materials for Efficient Bioethanol Production. Adv Biochem Engin/Biotechnol 2007; 108:41-65.

Giannino ML, Aliprandi M, Feligini M, Vanoni L, Brasca M, Fracchetti F: A DNA array based assay for the characterization of microbial community in raw milk. J Microbiol Methods 2009; 78:181-188.

Giuliodori AM, Gualerzi CO, Soto S, Vila J, Tavío MM: Review on Bacterial Stress Topics. Ann N Y Acad Sci 2007; 1113:95-104.

Hassan EA-E, Amr AE-H: Lignin Biodegradation with Ligninolytic Bacterial Strain and Comparison of *Bacillus subtilis* and *Bacillus sp.* Isolated from Egyptian Soil. Am-Euras J Agric Environ Sci 2009; 5:39-44.

Jadhav JP, Phugare SS, Dhanve RS, Jadhav, SB: Rapid biodegradation and decolorization of Direct Orange 39 (Orange TGLL) by an isolated bacterium Pseudomonas aeruginosa strain BCH. Biodegradation 2010; 21:453-463.

Kasana RC, Salwan R, Dhar H, Dutt S, Gulati A. A rapid and easy method for the detection of microbial cellulases on agar plates using gram's iodine. Curr Microbiol 2008; 57:503-507.

Kirk TK, Lundquist K: Comparison of sound and white-rotted sapwood of sweetgum with respect to properties of the lignin and composition of extractives. Sv Papperstidn 1970; 73:294-306.

Kirk TK: Effects of microorganisms on lignin. Annu Rev Phytopathol 1971; 9:185-210.

Maki M, Leung KT, Qin W: The prospects of cellulose-producing bacteria for the bioconversion of lignocellulosic biomass. Int J Biol Sci 2009; 5:500-516.

Maki M, Broere M, Leung KT, Qin W: Characterization of some efficient cellulase producing bacteria isolated from paper mill sludges and organic fertilizers. Int J Biochem Mol Biol 2011; 2:146-154.

Ng TK, Zeikus JG: Comparison of extracellular cellulase activities of clostridium thermocellum LQRI and trichoderma reesei QM9414. Appl Environ Microbiol 1981; 42:231-240.

Otjen J, Blanchette RA: Patterns of decay caused by Inonotius dryophilus (Aphyllophorales: Hymenochaetaceae) a white-pocket rot of oaks. Can J Bot 1982; 60:2270-2279.

Pandey KK, Pitman AJ: FTIR studies of the changes in wood chemistry following decay by brown-rot and white-rot fungi. Int Biodeterior Biodegrad 2003; 52:151-160.

Raj A, Chandra R, Reddy MMK, Purohit HJ, Kapley A: Biodegradation of kraft lignin by a newly isolated bacterial strain, *Aneurinibacillus aneurinilyticus* from the sludge of a pulp paper mill. World J Microbiol Biotechnol 2010; 23:793-799.

Ragauskas AJ, Williams CK, Davison BH, Britovsek G, Cairney J, Eckert CA, Frederick Jr. WJ, Hallett JP, Leak DJ, Liotta CL, Mielenz JR, Murphy R, Templer R, Tschaplinski T: The path forward for biofuels and biomaterials. Science 2006; 311: 484-489.

Rivas R, Trujillo ME, Mateos PF, Martínez-Molina E, Velázquez E: Cellulomonas xylanilytica sp. nov., a cellulolytic and xylanolytic bacterium isolated from a decayed elm tree. *Int J Syst Evol Microbiol* 2004; 54:533-536.

Scheller HV, Ulvskov P: Hemicelluloses. Annu Rev Plant Biol 2010; 61:263–289.

Schneider SH: The greenhouse effect: Science and policy. Science 1989; 243:771-781.

Sizova MV, Izquierdo JA, Panikov NS, Lynd LR. Cellulose- and Xylan-Degrading Thermophilic Anaerobic Bacteria from Biocompost. Appl Environ Microbiol 2011; 77:2282-2291.

Thivend S, Lebreton P: Champignons lignivores. Contribution a l'etude de la degradation de la lignine. Revue ATIP 1969; 23:13-24.

Xiao Z, Storms R, Tsang A: Microplate-based filter paper assay to measure total cellulase activity. Biotechnol Bioeng 2004; 88:832-837.

Table 1. The BLAST search results for the sequenced cellulase producing isolates.

	Isolate	Identity (%)	Likely genus
Municipal Waste	AS1	100	*Pseudomonas*
	AS2A	97	*Rhodococcus*
	AS2B	100	*Exiguobacterium*
	AS3	100	*Arthrobacter*
	AS4	100	*Microbacterium*
	CDS1B	99	*Aeromonas*
	CDS2A	97	*Chryseobacterium*
	CDS2B	100	*Bacillus*
	CDS3	100	*Pseudomonas*
	CTS1A	100	*Bacillus*
	CTS1B	100	*Bacillus*
	CTS2	100	*Bacillus*
	GH201	100	*Pseudomonas*
Peat	55S1	99	*Bacillus*
	55S2	98	*Duganella*
	55S5	100	*Bacillus*
	65S3	97	*Bacillus*
	65S5	96	*Paenibacillus*
	6S1	100	*Bacillus*
	6S4	100	*Duganella*

Figure Legend

Figure 1. Twenty cellulase-producing isolates grown on CMC agar for 48 h at 28°C and grouped based on A) bacteria isolated from peat and B) the bacteria derived from municipal waste. Additionally, a positive and negative control, *C. xylanilytica* and *E. coli* JM109, respectively, are included in B.

Figure 2. Phylogenetic tree produced from the alignments of 16S rDNA fragments from the isolates, presented in TreeView. Closer related isolates and their respective Phyla are outlined as indicated in the legend. The diameter of halos the isolates produced on CMC agar is respectively shown with a colour scale indicating small to large halos, qualitative cellulase activity.

Figure 3. Qualitative results for the 6 isolates grown at 28°C and shaking at 180 rpm, capable of completely degrading filter paper within 10 days incubation: A) 6S1 *Bacilus*, B) 55S5 *Bacillus*, C) positive control *C.* xylanilytica, D) negative control *E. coli*, E) AS1 *Pseudomonas*, F) CDS1B *Bacillus*, G) CH20S1 *Bacillus*, H) CTS1B *Bacillus*.

Figure 4. Xylanase positive isolates on xylan agar grown for 48 h at 28°C, shown by the appearance of halos after staining with Gram"s iodine solution. Grouped into A) bacteria isolated from peat and B) those isolated from municipal waste, including positive and negative controls (*C. xylanilytica* and *E. coli* JM1O9, respectively).

Figure 5. A) Change in colour (Abs_{425nm}) of 1% black liquor with recorded final pH and B) Survival of all isolates reported as cell density (log $CFUml^{-1}$), for all isolates which can tolerate 1% black liquor for 21 days incubation at 28°C, shaking 180 rpm. Isolates: AS1 (■), AS4 (♦), CH2OS1 (●), 65S3 (▲), CTS1B (□), CTS1A (◊), 65S5 (○), 55S1 (Δ), 55S5 (x), 6S1 (+).

Figure 6. The percentage change in reflectance at 280nm of 0.1% pure kraft lignin after 21 days incubation at 28°C, shaking at 180 rpm with black liquor tolerant isolates (■) and compared to untreated sample (■).

Figure 7. FTIR analysis of all potential lignin modifying isolates comparing percentage preference for hemicellulose and lignin over cellulose after growth 21 days growth with 1% barely straw at 28°C, shaking 180 rpm.
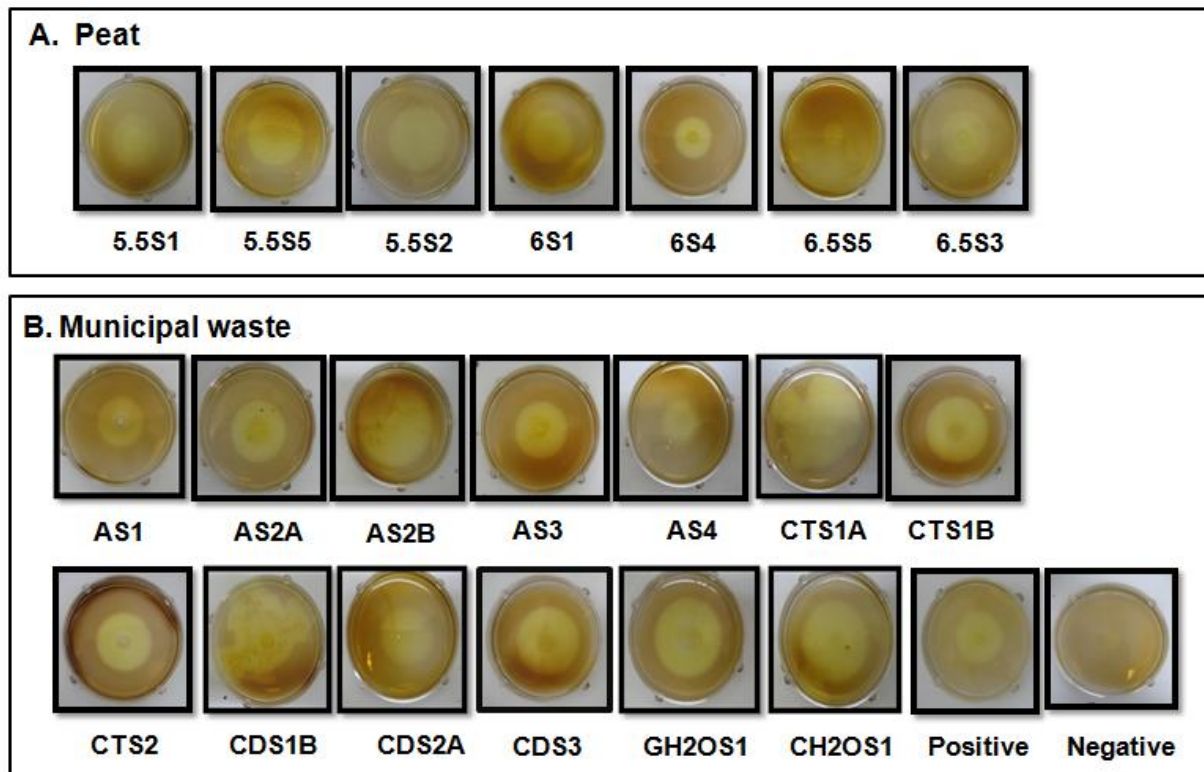
A. Peat

5.5S1 5.5S5 5.5S2 6S1 6S4 6.5S5 6.5S3

B. Municipal waste

AS1 AS2A AS2B AS3 AS4 CTS1A CTS1B

CTS2 CDS1B CDS2A CDS3 GH2OS1 CH2OS1 Positive Negative

*Controls: *C. xylanilytica* (positive) and *E. coli* JM1O9 (negative).

Figure 1

109

Figure 2
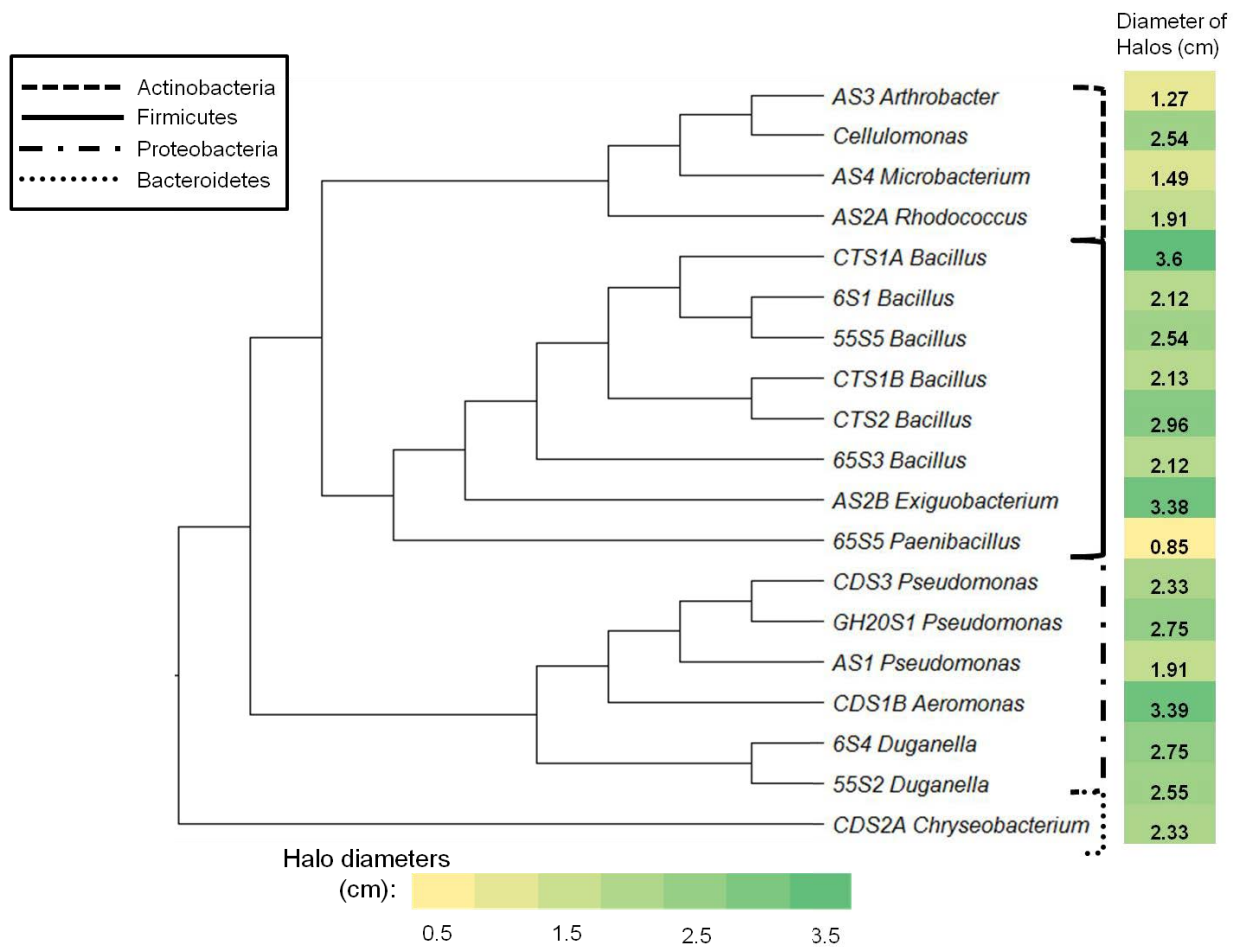
A.	B.	C.	D.	E.	F.	G.	H.
782.6± 123	978.3± 92	1043± 10	0±0	847.8± 31	913.0± 215	608.7± 245	1065± 92
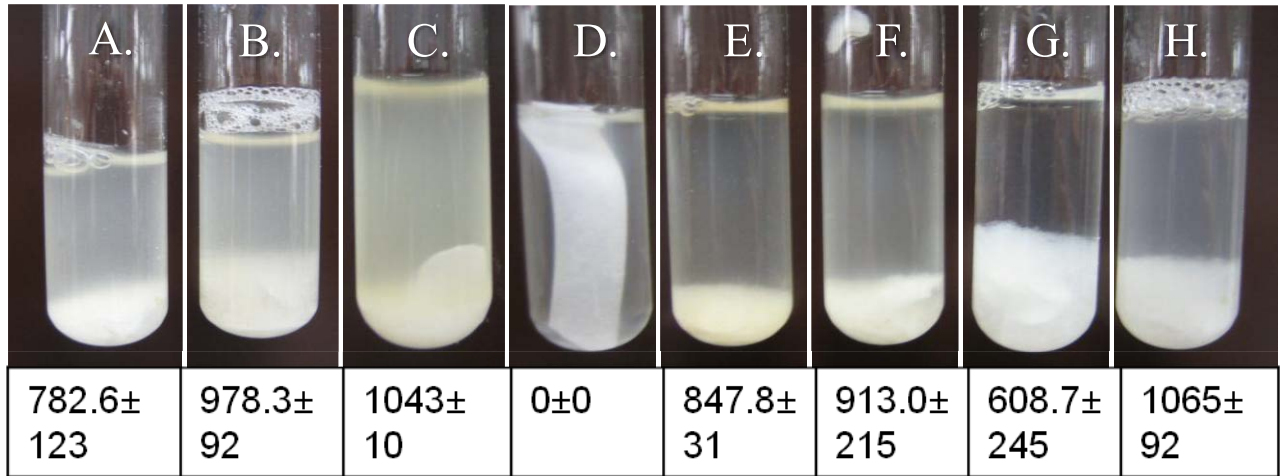
*(nM glucose equivalents after degradation)

Figure 3

A. Peat

6S1   5.5S1   5.5S2   5.5S5

B. Municipal waste

AS1   AS2A   AS2B   AS3   GH2OS1

CDS1B   CDS3   CTS1B   positive   negative
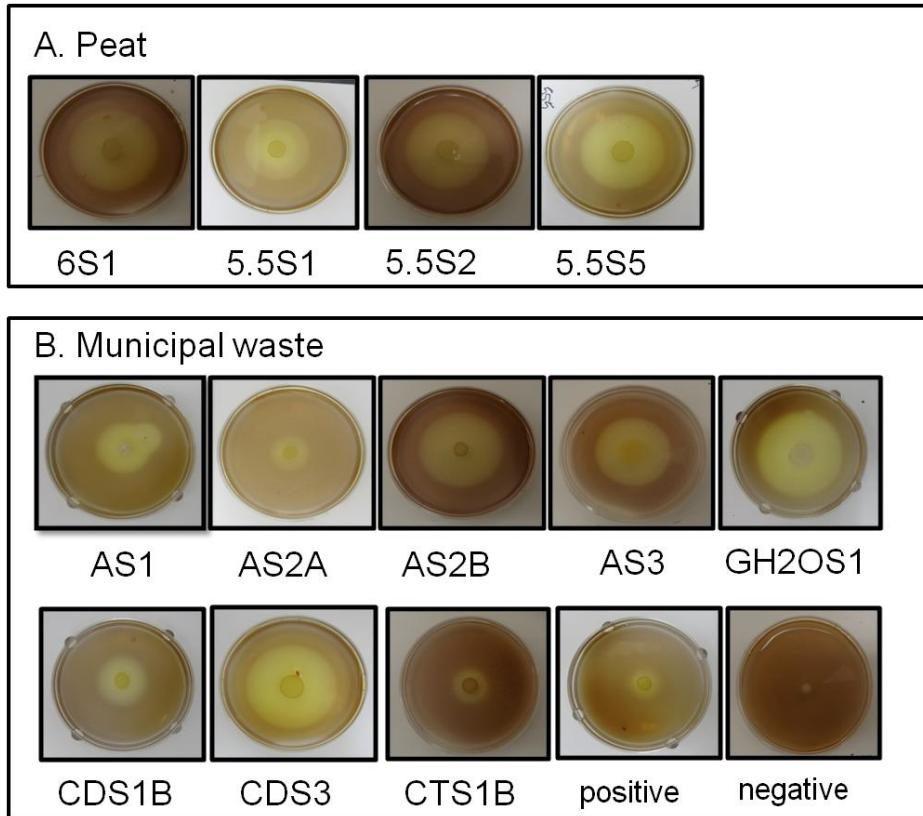
*Controls: *C. xylanilytica* (positive) and *E.coli* JM1O9 (negative).
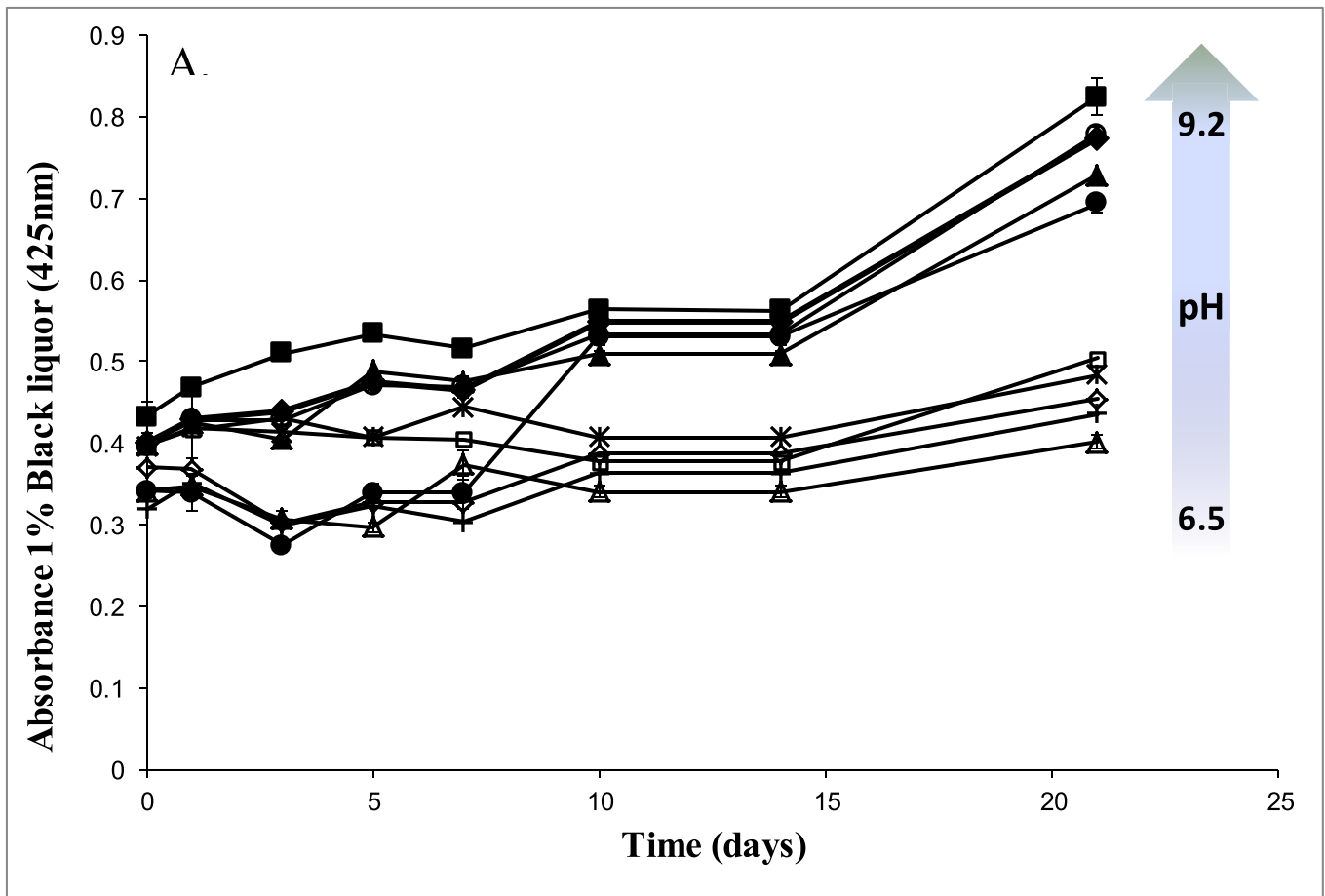
Figure 4

Figure 5A
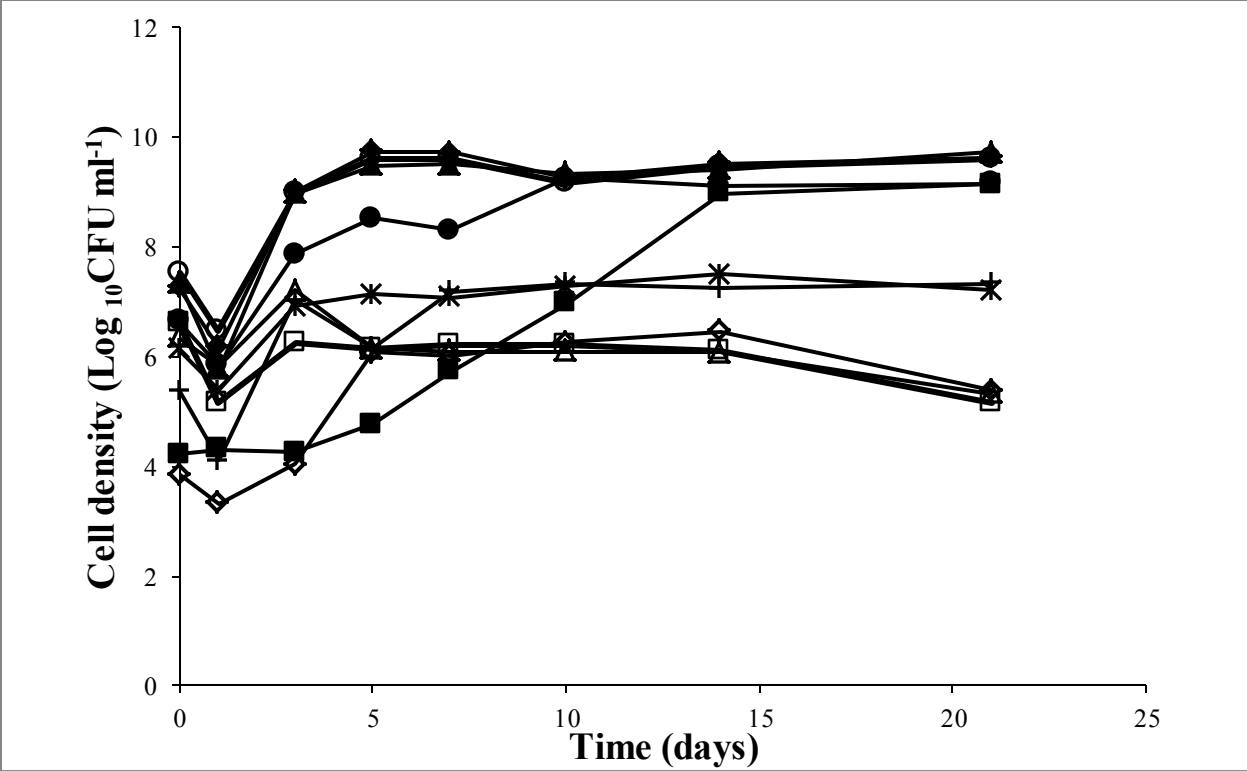
Figure 5B
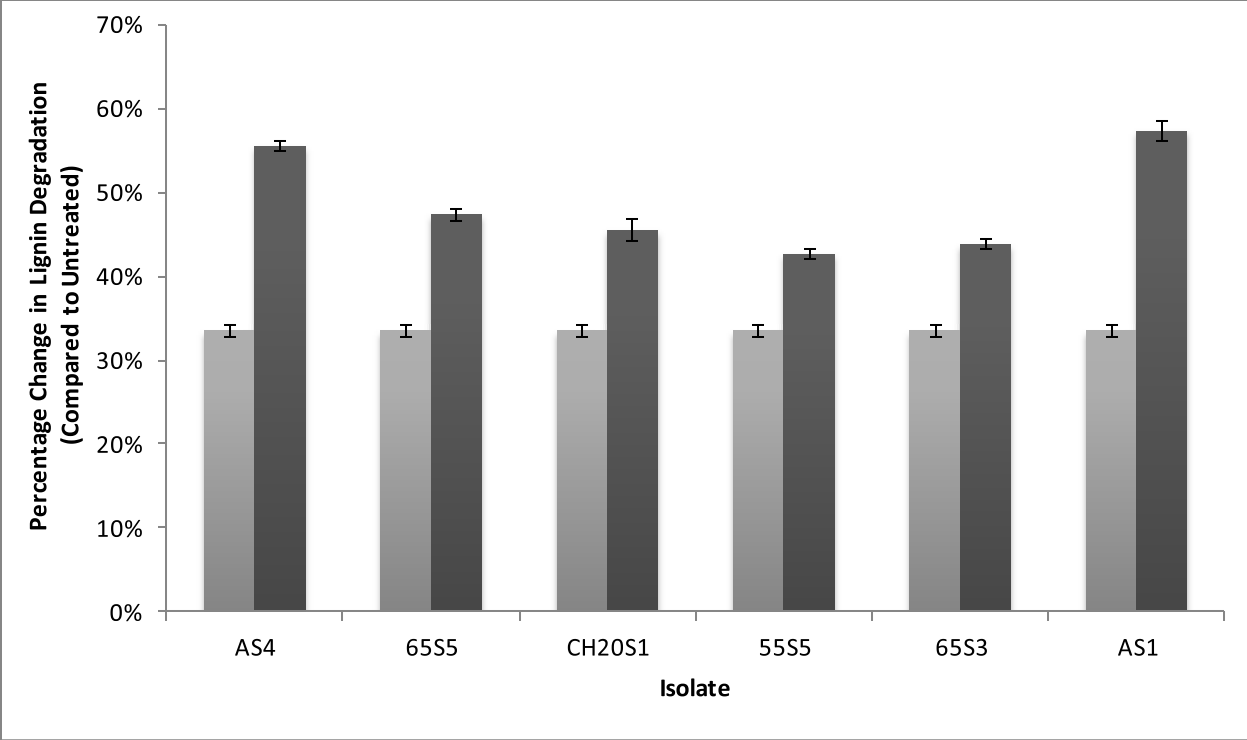
Figure 6
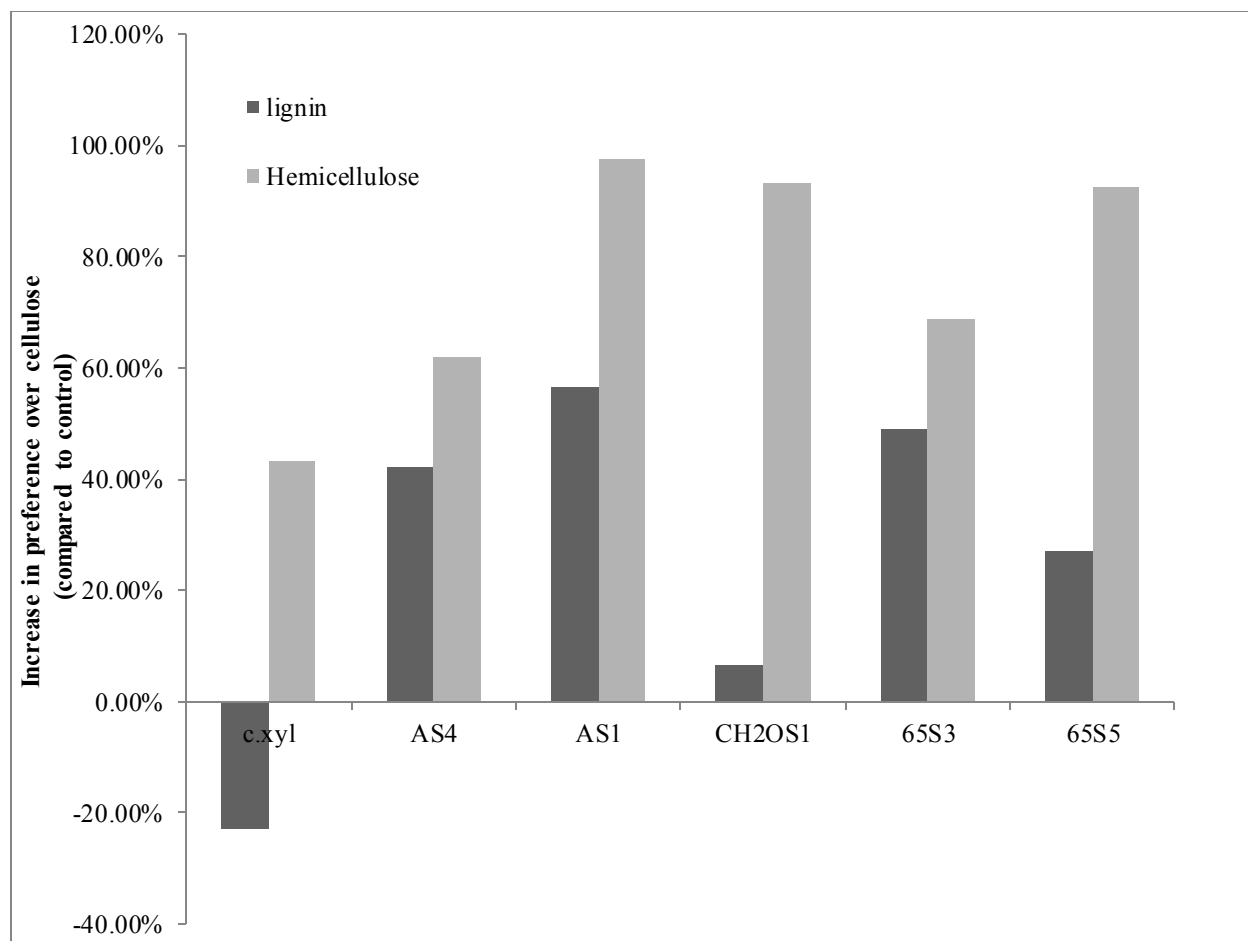
Figure 7

Chapter 4: Increased expression of β-glucosidase A in *Clostridium thermocellum* 27405 significantly increases cellulase activity

Authors: Miranda Maki, Lachlan Armstrong, Kam Tin Leung, Wensheng Qin

4.1 Abstract

β-glucosidase A (*bgl*A*)* in *Clostridium thermocellum* 27405 was increased by expression from shuttle vector pIBglA in attempts to increase cellulase activity and ethanol titre by lowering the end-product inhibition of cellulase. Through a modified electrotransformation protocol *C. thermocellum* transformant (+MC*bgl*A) harbouring pIBglA was produced. The β-glucosidase activity of +MC*bgl*A was 2.3- and 1.6-fold greater than wild-type (WT) during late log and stationary phases of growth. Similarly, total cellulase activity of +MC*bgl*A was shown to be 1.7-, 2.3- and 1.6-fold greater than WT during, log, late log and stationary phases of growth. However, there was no significant correlation found between increased cellulase activity and increased ethanol titres for +MC*bgl*A compared to the WT. *C. thermocellum* has industrial potential for consolidated bioprocessing (CBP) to make a more cost effective production of biofuels; however, the hydrolysis rate of the strain is still hindered by end-product inhibition. We successfully increased total cellulase activity by increased expression of *bgl*A and thereby increased the productivity of *C. thermocellum* during the hydrolysis stage in CBP. Our work also lends insights into the complex metabolism of *C. thermocellum* for future improvement of this strain.

4.2 Introduction

The production and commercialization of biofuels has gained a great deal of attention and support yet there remain some major bottlenecks with its current status; namely, there is a lack of biocatalysts that

can work efficiently at high temperatures and under extreme pH conditions. Moreover, few microorganisms produce all the required enzymes for efficient hydrolysis of hemicellulose and cellulose. Additionally, hydrolysis and fermentation of lignocellulosic biomass-derived sugars requires separate steps (i.e. fungal enzymes and *Saccharomyces cerevisiae*, respectively), which in turn is less cost-effective.[1] However, *Clostridium thermocellum* is a great potential candidate for biofuel production due to its ability to combine cellulase production and saccharification of biomass with fermentation in a process referred to as consolidated bioprocessing (CBP).[2] In addition, *C. thermocellum* is a Gram positive, anaerobic, thermophilic, ethanologenic and cellulosome-producing bacteria. This means that during the production of biofuels from cellulosic and hemicellulosic biomass, *C. thermocellum* could decompose the biomass using highly versatile cellulosomes in addition to free cellulases and hemicellulases, and ferment 6-carbon sugars to ethanol without the addition of oxygen. Also, the thermophilic nature, (growth optimum 60°C), would allow easier extraction of the ethanol which requires higher temperatures to volatilize and precipitate.[3] Currently, *C. thermocellum* has yet to be widely exploited in the production of biofuels due to several limiting factors. For example, *C. thermocellum* can hydrolyze both cellulose and hemicellulose; however, it can only ferment 6-carbon sugars and thus 5-carbon sugars are not being utilized. Additionally, end-products of fermentation such as lactic and acetic acids, as well as ethanol can be toxic to *C. thermocellum*. Also, stresses such as toxicity and oxygen exposure can cause sporulation because it is a spore-forming bacterium.[4,5] Further, cellulase activity in *Clostridium cellulolyticum* is shown to be inhibited by end-products such as cellobiose.[6] Inhibition of cellulase activity ultimately represents one of the greatest limitations to using *C. thermocellum* for biofuels. Without maximum hydrolysis of cellulosic biomass we cannot begin to consider optimum ethanol production and we cannot begin to change the economic viability of biofuels. Researchers have suggested and shown that by adding exogenous β-glucosidase isolated from *Aspergillus niger* one can increase the total cellulase activities of the cellulosome from *C. thermocellum* by 10-fold, in vitro.[7] However, the purification and then addition of exogenous β-glucosidase would not be cost-effective for large-scale biofuel production. The genetic

modification of the thermophilic anaerobe *C. thermocellum,* has been limited due to the strict restriction endonuclease system, which is described as a Dam+ phenotype.[8,9] Also, many Gram positive bacteria with their thick cell walls have been reported as difficult to electrotransform.[10,11] In this study, we have increased the copy number of β-glucosidase A gene (*bglA*) in *C. thermocellum* 27405 by electrotransforming it with a newly constructed shuttle vector (pIBglA) to ultimately increase cellulase activity and evaluate the overall effects on valuable end-product formation such as ethanol.

4.3 Materials and Methods

4.3.1. Media, strains and cultivation conditions

The strain *Clostridium thermocellum* (ATCC 27405) was obtained from the American Type Culture Centre through Cedarlane in Canada. *C. thermocellum* cells were grown at 60°C in the chemically defined medium *Clostridium thermocellum* Medium (ATCC media 1191), which according to the ATCC protocol contains a mineral elixir, reducing solution and vitamin solution. Either cellobiose or Avicel were included as the carbon source for appropriate experiments at a concentration of 0.5% and 1%, respectively.[12,13] The following antibiotics were used for all growth, experiments transformant to maintain selection: 100 μg/ml ampicillin and 20 μg/ml lincomycin (Sigma-Aldrich, Canada). All of the work in this study using *C. thermocellum* cells was done inside a Coy Anaerobic Chamber (Coy Laboratories, USA) under 5% hydrogen, 95% nitrogen mixed atmosphere, except applying the electric potential to the cells, centrifugation of cells and genomic DNA or enzyme extraction. The other strain used in this study *Escherichia coli* JM1O9, was grown at 37°C in Luria Bertani broth or on Luria Burtani agar containing 100 μg/ml of ampicillin for selection of transformants when appropriate.

4.3.2. Cloning β-glucosidase A from C. thermocellum

The genomic DNA of *C. thermocellum* was extracted from 3 ml of 48 h broth cultures using the Fungi/Yeast Genomic DNA Isolation Kit (Norgen Biotek Corporation, Canada) according to the instructions for Gram positive bacteria provided by the supplier. Two primers, BglPFW and BglSRV (shown in table 1), were used to amplify the complete sequence of *bgl*A from *C. thermocellum* with promotor and temerinator. The primers were designed to contain the restriction cut sites for *Pst*I and *Sac*I for BglPFW and BglSRV, respectively, in addition to nucleotides complementary to *bgl*A. The PCR reaction mixtures contained 10 ng of *C. thermocellum* genomic DNA, 10 pmol of both forward and reverse primers, 10x Taq buffer with 500 mmol KCl, 25 mmol/l $MgCl_2$, 0.2 mmol deoxynucleoside triphosphate, and 5 U *Pfu* DNA polymerase per 50 μl reaction. The PCR program was as follows: primary denaturation 3 minutes at 95°C, followed by 35 amplification cycles consisting of denaturing at 95°C for 1 minute, annealing for 1 minute at 54°C, and extension at 72°C for 1 minute, upon completion of 35 amplification cycles a final extension step was done at 72°C for 10 minutes. The resulting amplicon of approximately 1.3 kb was confirmed by sequencing on ABI 3730xl automatic sequencer (Eurofins MWG Operon, Canada). The complete *bgl*A was confirmed by complementation and alignment of sequencing results using DNAMAN software. The *bgl*A product was then digested with restriction enzymes *Pst*I and *Sac*I (Fermentas, Canada) by combining 5 μl of *bgl*A PCR product with 0.6 μl of *Sac*I and *Pst*I and 2 μl of 10x Tango buffer, incubated for 3 h 37°C.

4.3.3. Source and construction of plasmid pIBglA

Plasmid pIKm1 was a gift from Lee Lynd (Dartmouth College, USA). The pIKm1 DNA was isolated from *E. coli* using Ultra Clean 6 Minute Mini Plasmid Kit (Mo Bio Laboratories, Canada) following the directions provided by the supplier. No sequence information was available for plasmid pIKm1 therefore we designed the primers KmFW and KmRV, (Table 1). The primers were designed within the kanamycin cassette gene to sequence the flanking multiple cloning sites. Restriction maps were produced from

resulting sequences using DNAMAN software and cross referenced to the sequence of *bglA*. Two restriction endonucleases were chosen and used for digestion of pIKm1: *Pst*I and *Sac*I (Fermentas, Canada), restriction digest was performed by combining 0.8 μg/μl of pIKm1 DNA with 6.3 U of *Sac*I and 2 μl of 10X Tango Buffer (Fermentas), this mixture was then incubated at 37°C in a water bath for 1 h. After 1 h time, 6.3 U of *Pst*I was added and then the mixture was incubated for an additional 2 h at 37°C. Digestion resulted in two bands, one approximately 1.5 kb and the other approximately 5 kb. The 5 kb representing the remainder of the vector minus most of the kanamycin cassette was gel extracted using the NucleoSpin Extraction II kit (Clonetech Laboratories, Canada) following instructions provided by the distributor. Previously cloned, digested and cleaned *bgl*A were ligated to the approximate 5 kb vector using T4 DNA ligase (Fermentas, Canada). The ligation reaction mixture contained 2 μl of ligation buffer, 5 U T4 DNA ligase, 15 μl of 80 ng μ/l *bgl*A DNA and 2 μl of 100 ng μ/l of digested pIKm1 DNA, and allowed to incubate at ambient temperature for 3 hr. After ligation, the resulting DNA was transformed to *E. coli* JM1O9 using 40 μl of prepared electrocompetent cells premixed with 1 μl of ligation reaction in a 0.4 cm cuvette at a capacitance of 25 μF using the Bio- Rad Gene Pulser apparatus (Bio-Rad Laboratories, Canada). Transformants were screened using colony PCR for the presence of two genes: *bgl*A and ampicillin (*amp*) (Table 1).

4.3.4. Electrotransformation of C. thermocellum with pIBglA

Electrocompetent *C. thermocellum* cells were prepared precisely following the method described by Tyurin *et al.* in 2004, however no isoniacin was used.[14] Final cell suspension was approximately 9×10^{10} cells/ml and 40 μl aliquots were divided into 0.4 cm gap electroporation cuvettes and chilled on ice for 5 minutes. One microlitre of ~2 μg of pIBglA DNA extract was premixed under anaerobic conditions with the chilled 40 μl cells. The cuvettes with cell + DNA on ice were removed from the anaerobic chamber 120 and immediately place under a constant stream of N_2 where the Bio-Rad Gene Pulser apparatus was used to electroporate the cells at a capacitance of 25 μF. Still under N_2, the cells were then immediately

removed from the cuvette with a 1 ml syringe and injected through the rubber stopper of hungate tubes (Bellco Glass, Canada) in prewarmed (55°C) *C. thermocellum* media supplemented with 0.5% (w/v) cellobiose and 0.75% (w/v) agar and 100 µg/ml ampicillin and 20 µg/ml lincomycin. PCR was carried out with primers designed for the ampicillin gene (Table 1) using total DNA from the ampicillin-lincomycin resistant clones as a template. To determine the approximate stability of the vector, cell densities were measure by reading O.D.600nmm absorbance readings every 24th hour with the the addition of antibiotics to *C. thermocellum* medium over 8 subcultures and without the addition of antibiotics for 8 generations in separate triplicate experiments.

4.3.5. *β-glucosidase activity of wild-type C. thermocellum and C. thermocellum+MCbglA*

The β-glucosidase activity of wild-type- (WT-) *C. thermocellum* and the transformant containing multiple copies of *bgl*A (MC*bgl*A-*C. thermocellum*) was assayed by measuring the increase of absorbance at 400nm via the release of *p*-nitrophenol from *p*-nitrophenyl-β-D-glucopyranoside (PNPG).[15] Briefly, WT- and MC*bgl*A- *C. thermocellum* were pre-cultured for 48 h in 6 ml of *Clostridium* broth with 1% (w/v) cellobiose as the sole carbon source (n=3). Then 200 µl of each culture were subsequently inoculated in triplicate to 40 ml of the same broth. Growth was monitored over 48 h during batch culture and 3 ml of samples were collected for each strain at early exponential phase (0.25 O.D.600nm), late exponential phase (0.5 O.D.600nm) and stationary phase (0.7 O.D.600nm) in triplicate for enzyme analysis. To extract total cellular β-glucosidase, cells were harvested at 17,000 *g* for 1 min; then washed twice with chilled 100 mM PBS (pH 7.0) before final resuspension in 1 ml of PBS. Microbeads were filled to 0.5 µl mark of the 1.5 ml microcentrifuge tubes then samples were vortexed at full speed for 5 min, then chilled in an ice bath for 5 min; this procedure was repeated 2 more times for a total of 15 min vortexing. Following, the samples were centrifuged at 17,000 *g* for 1 min. In a 96 well microtitre plate, 50 µl of 100 mM PBS (pH 7.0) containing 4 mM PNPG was loaded per well, plus the addition of 50 µl of enzyme extract for each strain and each growth phase in triplicate. The microtitre plate was then incubated at

55°C for 30 min. Thereafter, the reaction was stopped by adding 100 µl of chilled 1M Na_2CO_3, followed with a 10 min incubation at 4°C. The absorbance was measured on a Bio-Rad Laboratories xMark spectrophotometer at 400 nm. The β-glucosidase activity was defined as the amount of p-nitrophenol (PNP) released per 30 min incubation.[15]

4.3.6. Total cellulase activity of wild-type and +MCbglA C. thermocellum

Total cellulase activity was defined as the amount of glucose and cellobiose released from 1% Avicel and expressed in glucose equivalents (µM). Wild-type *C. thermocellum* (WT) and *C. thermocellum*+MC*bgl*A (+MC*bgl*A) were cultured for 48 h in 6 ml of *C. thermocellum* media supplemented with 1% Avicel (w/v) as the sole carbon source (n=3), then 200 µl of each strain was subsequently transferred in triplicate to a fresh new 40 ml of the same media for batch cultures. Samples were collected in 1.5 ml aliquots from each vial to determine total cellulase activity during exponential (0.25 O.D.600nm), late exponential (0.5 O.D.600nm) and stationary (0.7 O.D.600nm) phases of growth. A microplate assay using the di-nitrosalicylic acid (DNS) method to measure reducing sugars, modified from Xiao et al.[16] was used. Briefly, 60 µl of supernatant from each strain during each growth phase was added to a well in triplicate, a 120 µl of DNS was also added to each well. The plate was then incubated at 95°C for 5 min. Finally, 36 µl was removed from each well and added to 160 µl of ddH_2O, mixed, and then the absorbance was read on a Bio-Rad xMark spectrophotometer at 545 nm.

4.3.7. Analytical methods for ethanol

Broth samples for ethanol analysis were collected from batch cultures used to measure enzyme activities from trials of WT- and +MC*bgl*A- *C. thermocellum* on both cellobiose and Avicel containing media previously mentioned, by aliquoting 1.5 ml of culture supernatant into 1.5 ml Eppendorf tubes. All samples were stored at -20°C until analysis. The samples were then analyzed using an Agilent 6850 Gas

Chromatograph fitted with a Carbowax column (30m x 0.32 mm, film thickness 0.25 μm) and flame ionization detector. In preparation for analysis, the samples were thawed at room temperature, centrifuged at 17,000 g for 5 min and 1 ml of each sample was transferred to a fresh 1.5 ml Eppendorf tube. Prior to injection, each sample was spiked with 100 μl n-butanol, which acted as an internal standard. Injection volume was 1μl and the inlet was run splitless. Nitrogen was used as a carrier gas with a flow rate of 1.5 ml/min and the run time was 5 min/sample. The injection port temperature was set at 250°C, the column temperature was isothermally set at 75°C, and the detector temperature was 300°C. Standards were prepared the day of analysis using anhydrous ethanol and they were also spiked with 100 μl n-butanol per 1 ml standard.

4.4 Results

4.4.1. Construction of plasmid pIBglA

Plasmid pIBglA was constructed and transformed into Dam+ *E. coli* JM109. Due to the enzymatic limitations observed during experiments using SacI, which according to the distributer notes has several inhibitors causing low cutting efficiency, transformation of ligation products resulted in 1 positive *bgl*A-containing transformant from 68 ampicillin positive transformants. Plasmid extraction of pIBglA followed by 1% agarose gel electrophoresis confirmed an approximate size of 7 kb. Sequencing revealed the complete cloned sequence of *bgl*A of approximately 1,800 bp.

4.4.2. Verification of C. thermocellum electrotransformation with pIBglA

The transformation of *C. thermocellum* with pIBglA was completed and selection was based on a combined resistance to appropriate concentrations of ampicillin and lincomycin in semi-solid agar. Electrocompetent *C. thercmocellum* cells were transformed at a rate of 5.17±3 transformants ml-1 of *C. thermocellum* media agar supplemented with appropriate concentrations of ampicillin and lincomycin. Thus, strain *C. thermocellum*+MC*bgl*A (+MC*bgl*A) was created. No spontaneous ampicillin-lincomycin

resistant *C. thermocellum* cells were detected after 6 days incubation. PCR was carried out as shown in Figure 1, the presence of the ampicillin gene resulted in a ~530 bp product (lane 2), confirmed by pIBglA plasmid DNA as a positive control (lane 3) and negative control (WT total DNA) (lane 4). Growth in ampicillin exhibited the same growth rate upon subuculturing; however, the loss of ampicillin resistance when continuously subcultured without antibiotics was observed at approximately the 5th generation as was observed through O.D.$_{600nm}$ absorbance readings (data not shown here).

4.4.3. *β-glucosidase activity of wild-type C. thermocellum and C. thermocellum+MCbglA*

The β-glucosidase activity, was evaluated for WT- and +MC*bgl*A- *C. thermocellum* and was found to be an average 1.9-fold greater in +MC*bgl*A directly correlating with an increase in expression of β-glucosidase from plasmid pIBglA. As seen in Figure 2, the β-glucosidase activity increased during late log and stationary phases of growth for +MC*bgl*A and were found to be 2.3- and 1.6-fold greater than WT with a statistical significance of $p<0.05$ (Student"s *t* test). However, biological and technical replicates revealed there was no significant difference in β-glucosidase activity during early log phases of growth for WT and +MC*bgl*A.

4.4.4. *Total cellulase activity of wild-type and +MCbglA C. thermocellum*

The total cellulase activity, the amount of glucose equivalents released (μM) from 1% Avicel, of WT- and +MC*bgl*A- *C. thermocellum* was evaluated to determine if an increase in expression of *bgl*A could increase the overall cellulase activity during batch culture trials. The results in Figure 3 show that total cellulase activity of the +MC*bgl*A was observed to be 1.7-, 2.3- and 1.6-fold significantly greater than the activity of WT during log, late log and stationary phases of growth, respectively, $p<0.05$ (Student"s *t* test). Thus, total cellulase activity was an average 1.9-fold greater for +MC*bgl*A compared to the WT.

4.4.5. Ethanol analysis for wild-type and +MCbglA C. thermocellum

WT- and +MC*bgl*A- *C. thermocellum* were grown in 1.5% cellobiose and 1% Avicel medium. Samples were taken when the cultures reached log and stationary phases of growth and analyzed for ethanol concentration (Figure 4). When +MC*bgl*A reached stationary phase, the average ethanol concentration in the media was 2.5 g/l. This is slightly higher than what was observed for the WT *C. thermocellum* (1.9 g/l), but this difference was deemed not to be statistically significant. Likewise, +MC*bgl*A produced more ethanol during log phase growth, but it was not significantly different from the WT. Ethanol production by +MC*bgl*A was also investigated in Avicel medium, and it yielded similar results to the cellobiose trials. No significant differences were observed between the *bglA* copy number mutant and the WT in both the log and stationary phases. Little difference was observed between ethanol concentrations at log and stationary phases.

4.5 Discussion

The opportunity to use *C. thermocellum* for CBP in the biorefining industry has exceptional potential if we can overcome some of the challenges facing its development. The majority of difficulty working with thermophilic anaerobic bacterial systems arises from the slow progress in genetic manipulation of these systems. Plasmid pIKM1 bearing a kanamycin cassette and with Gram negative and positive origins of replication was constructed by Mai et al. in 1997 and transformed into *Thermoanaerobacterium* sp. strain JW/SL-YS485 a close thermophilic anaerobic strain to *C. thermocellum*.[17] Then, it was not until 2004, that Tyurin et al. developed an efficient protocol to transform *C. thermocellum* 27405 among two other *C. thermocellum* strains DSM 1313 and 4150 with plasmid pIKM1 using a uniquely designed electroporation system.[14] More recently in 2010, Lin et al. developed a minimally invasive ultrasound-based sonoporation method for simple and rapid transformation of thermophilic Gram positive anaerobes.[18] In doing so, they transformed *Thermoanaerobacterium* sp. strain X514 with pIKM2

harboring a *C. thermocellum* β-1-4- glucanase gene, endoglucanase activity was observed in both electroporated and sonoporated X514 samples. Now for one of the first times reported, we expressed a functional gene (β -glucosidase A) from a Dam methylated plasmid pIBglA (constructed from pIKM1) in *C. thermocellum* 27405 using a modified electroporation protocol. In this research article, we strived to develop a *C. thermocellum* strain which can have greater total cellulase activity by reducing substrate specific end-product inhibition. Thus, we proposed increasing the copy number of β-glucosidase. The BglA gene was chosen because it is fully sequenced and also because it"s native to *C. thermocellum*. Thus, this gene is suitable for expression in its thermophilic host. Due to the current lack of confident recombinant systems for *C. thermocellum* and for a concern in disruption of vital genes we chose to use plasmid pIKM1 shuttle vector with low copy number to increase expression of BglA without overburdening the cell.

One of the greatest limitations to using any whole microorganism for hydrolysis of cellulose and hemicellulose is the end-product inhibition. Cellobiose is an inhibitor of cellulase activity in *C. thermocellum* and a previous report showed that the exogenous addition of β-glucosidase purified from *Aspergillus niger* could increase cellulase activity by 10-fold in *C. thermocellum*.[7] Moreover, in *Trichoderma reesei* transformants the heterologous expression of a β-glucosidase gene from *Penicillium decumbens* resulted in an average 30% increase in filter paper activity (representing total cellulase activity).[19] Therefore one may hypothesize that if there is an increase in copy number of β-glucosidase in *C. thermocellum*, there would also be an increase in β-glucosidase activity and this would ultimately increase total cellulase activity. In this study, the increase in copy number of *bgl*A significantly increased both β-glucosidase activity as well as total cellulase activity during late log and stationary phases of growth by an average of 2.0-fold and 1.9-fold, respectively, for +MC*bgl*A *C. thermocellum* over WT. Thus it appears the increase in β-glucosidase activity of *C. thermocellum* is also proportional to the observed increase in total cellulase activity. However, it was hypothesized here that the increase in total

cellulase activity would also result in a likewise increase in ethanol production during batch fermentation trials, due to an increase in glucose catabolism.

Nonetheless, there was no significant difference in ethanol production between WT- and +MC*bgl*A- *C. thermocellum* suggesting that total cellulase activity is not directly proportional to fermentative metabolism of glucose under batch fermentation conditions. We suggest that this could be due to a metabolic overburden in +MC*bgl*A from the presence of the shuttle vector pIBglA. This could potentially decrease the resistance of +MC*bgl*A to toxic end-products such as ethanol, lactic and acetic acids. The toxicity of end-products such as ethanol in the fermentation of glucose for ethanogenic microorganisms such as *C. thermocellum* has gained a lot of attention in research for biotechnological implications. Ethanol is known to inhibit glycolytic enzymes and cause damage to cell membranes, thus inhibiting cell growth.[20,21] From a commercial perspective titres of ethanol greater than 40 g/l are desired, for cost effective recovery.[22] Thus, researchers have found the need to develop greater ethanol tolerant *C. thermocellum* strains such as by adaptation to levels as high as 50-55 g/l.[22,23] However, to date, there remains a discrepancy whether increased ethanol tolerance can attribute to greater ethanol titres. The research presented here, also explored whether increased cellulase production could lend to an increase in ethanol. However, due to the lower levels of ethanol reported in this study, we suggest +MC*bgl*A strain be produced in the future using recombinant DNA techniques to eliminate the stress of maintaining a plasmid.

Anaerobic catabolism is a challenging yet refined process in *C. thermocellum* where the modest availability of ATP needs to support growth, cellulase production and the uptake; a complex process which still requires exploration.[24,25] It has been shown that the uptake of oligosaccharides with the combined intracellular phosphorolytic cleavage of β-1,4-glucosidic bonds is bioenergetically favourable when *C. thermocellum* is specifically grown on cellulose.[25] Despite the lack of regeneration of ADP co-factors produced through the hydrolytic activity of BglA, a greater overall cellulase activity could be

observed in +MC*bgl*A compared to the WT due to a reduction in the cellulase end-product inhibitor cellobiose. Additionally, the lack of increased ethanol production shown here further lends insights into the complex metabolism of glucose in *C. thermocellum*.

4.1 Conclusion

Our work clearly demonstrates that by increasing expression of native BglA there will be a nearly proportion increase in total cellulase activity of *C. thermocellum* leading to the production of a more industrially applicable *C. thermocellum*. The hydrolysis step is a rate limiting step in the production of biofuels; however, more work on +MC*bgl*A strain is required to increase ethanol titre for CBP potential. We will continue to test the following hypothesis: adapted increased tolerance to toxic end-products of +MC*bgl*A will lead to increased ethanol production, in hopes to lend more insight into the application of *C. thermocellum* in biofuel production.

References

1. Wyman CE, Dale BE, Elander RT, Hotzapple M, Ladisch MR, Lee YY. Coordinated development of leading biomass pretreatment technologies. Bioresour Technol 2005; 96:1959-66.

2. Maki M, Leung KT, Qin W. The prospects of cellulase-producing bacteria for the bioconversion of lignocellulosic biomass. Int J Biol Sci 2009; 5:500-16.

3. Demain AL, Newcomb M, Wu JHD. Cellulase, Clostridia, and Ethanol. Microbiol Mole Biol Rev 2004; 69:124–54.

4. Ng TK, Ben-Bassat A, Zeikus JG. Ethanol Production by Thermophilic Bacteria: Fermentation of Cellulosic Substrates by Cocultures of *Clostridium thermocellum* and *Clostridium thermohydrosulfuricum*. Appl Environ Microbiol 1981; 41:1337-43.

5. Mori Y. Characterization of a Symbiotic Coculture of Clostridium thermohydrosulfuricum YM3 and Clostridium thermocellumYM4. Appl Environ Microbiol 1990; 56:37-42.

6. Petitdemange E, Tchunden T, Valles S, Pirson H, Raval G, Gay R. Effect of carbon sources on cellulase production by *clostridium cellulolyticum* Biomass Bioenerg 1996; 3:393-402.

7. Lamed R, Kenig R, Morgenstern E, Calzada JF, De Micheo F, Bayer EA. Efficient cellulose solubilisation by a combined cellulosome-β-glucosidase system. Appl Biochem Biotechnol 1990; 27:173-83.

8. Klapatch TR, Demain AL, Lynd LR. Restriction endonuclease activity in Clostridium thermocellum and Clostridium thermosaccharolyticum. Appl Microbiol Biotechnol 1996; 45:127-31.

9. Ozkan M, Desai SG, Zhang Y, Stevenson DM, Beane J, White EA, et al. Characterization of 13 newly isolated strains of anaerobic, cellulolytic, thermophilic bacteria. J Ind Microbiol Biotechnol 2001; 27:275-80.

10. Dunny GM, Lee LN, LeBlanc DJ. Improved electroporation and cloning vector system for gram-positive bacteria. Appl Environ Microbiol 1991; 57:1194–201.

11. Löfblom J, Kronqvist N, Uhlén M, Ståhl S, Wernérus H. Optimization of electroporation-mediated transformation: Staphylococcus carnosus as model organism. J Appl Microbiol 2007; 102:736-47.

12. Brener D, Johnson BF. Relationship Between Substrate Concentration and Fermentation Product Ratios in Clostridium thermocellum Cultures. Appl Environ Microbiol 1984; 47:1126–9.

13. Bernardez TD, Lyford KA, Lynd LR. Kinetics of the extracellular cellulases of *Clostridium thermocellum* acting on pretreated mixed hardwood and Avicel. Appl Microbiol Biotechnol 1994; 41:620-5.

14. Tyurin MV, Desai SG, Lynd LR. Electrotransformation of *Clostridium thermocellum*. Appl Environ Microbiol 2004; 70:883-890.

15. Hang YD, Woodams EE. Apple pomace: A potential substrate for production of β-glucosidase by Aspergillus foetidus. Food Sci Technol 1994; 27:587-589.

16. Xiao Z, Storms R, Tsang A. Microplate-based filter paper assay to measure total cellulase activity. Biotechnol Bioeng 2004; 88:832-837.

17. Mai V, Lorenz WW, Wiegel J. Transformation of *Thermoanaerobacterium* sp. strain JW/SL-YS485 with plasmid pIKM1conferring kanamycin resistance. FEMS Microbiol Lett 1997; 148:163-167.

18. Lin L, Song H, Ji Y, He Z, Pu Y, Zhou J, Xu J. Ultrasound-Mediated DNA Transformation in Thermophilic Gram-Positive Anaerobes. PLoS one 2010; 5:1-10.

19. Ma L, Zhang J, Zou G, Wang C, Zhou Z. Improvement of cellulase activity in *Trichoderma reesei* by heterologous expression of a beta-glucosidase gene from *Penicillium decumbens*. Enzyme Microb. Technol 2011; 49:366-371.

20. Ingram LO. Ethanol tolerance in bacteria. CRC Crit Rev Biotechnol 1990; 9:305-319.

21. Jones RP. Biological principles for the effects of ethanol. Enzymol Microb Technol 1989; 11:130-151.

22. Shao X, Raman B, Zhu M, Mielenz JR, Brown SD, Guss AM, et al. Mutant selection and phenotypic and genetic characterization of ethanol-tolerant strains of *Clostridium thermocellum*. Appl Microbiol Biotechnol 2011; 92:641-652.

23. Williams TI, Combs JC, Lynn BC, Strobel HJ. Proteomic profile changes in membranes of ethanol-tolerant Clostridium thermocellum. Appl Microbiol Biotechnol. 2007; 74:422–32

24. Strobel HG, Caldwell FC, Dawson KA. Carbohydrate transport by the anaerobic thermophile *Clostridium thermocellum* LQRI. Appl Environ Microbiol 1995; 61:4012-4015.

25. Zhang P, Y-H, Lynd LR. Cellulose utilization by *Clostridium thermocellum*: Bioenergetics and hydrolysis product assimilation. PNAS 2005; 102:7321–7325.

Table 1. Primers used in this study.

DNA target	Forward 5''-3''	Reverse 5''-3''	Product (bp)
BglA	BglPFW-ACA<u>GAGCTC</u>AGCTCCATTGTTGCTTAGC	BglSRV-AAT<u>CTGCAG</u>ACTGGTAAGTGATTGCCGG	1867
pIKM1	KmFWD- CTGGGAAGAAGACACTCCA	KmRV- TGGAGTGTCTTCTTCCCAG	N/A
Ampicillin	AmpFW- CGTTCATCCATAGTTGC	AmpRV- GCACGAGTGGGTTACATCG	534

Figure legend

Figure 1. PCR amplification for confirmation of ampicillin fragment (~500bp). Lanes: 1. 1kb ladder, 2. +MCbglA total DNA, 3. plasmid pIbglA DNA (positive control), 4. WT total DNA (negative control).

Figure 2. The β-glucosidase activity of WT (■) and MC*bgl*A (■) during log, late log and stationary phases of growth and expressed as the amount of *p*-nitrophenol (μM) released after 30 min incubation with 4 μM PNPG.

Figure 3. The total cellulase activity of WT (■) and MC*bgl*A (■) during log, late log and stationary phases of growth and expressed in glucose equivalents (μM) released from 1% Avicel.

Figure 4. The ethanol titres (g l^{-1}) of WT (■) and MC*bgl*A (■) produced from batch ferementation trials on cellobiose and avicel containing mediums.
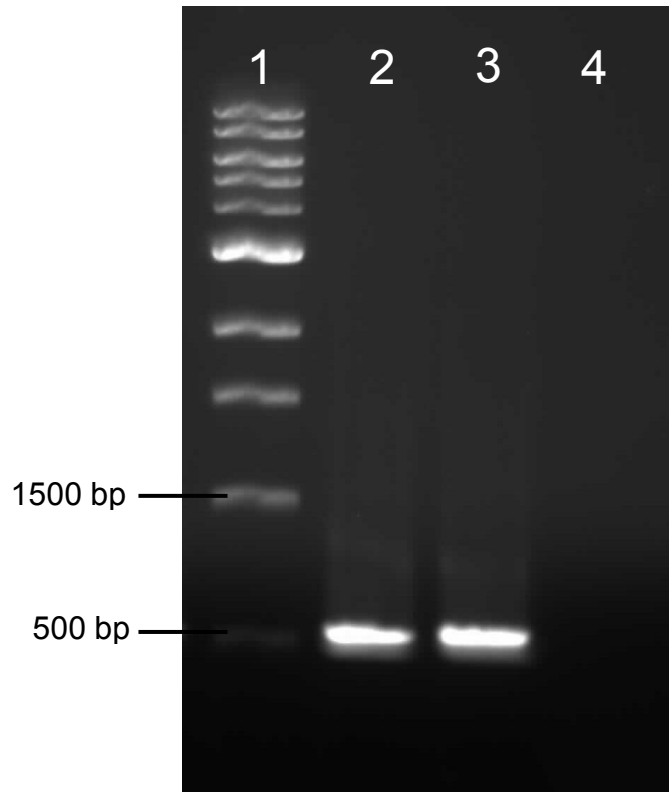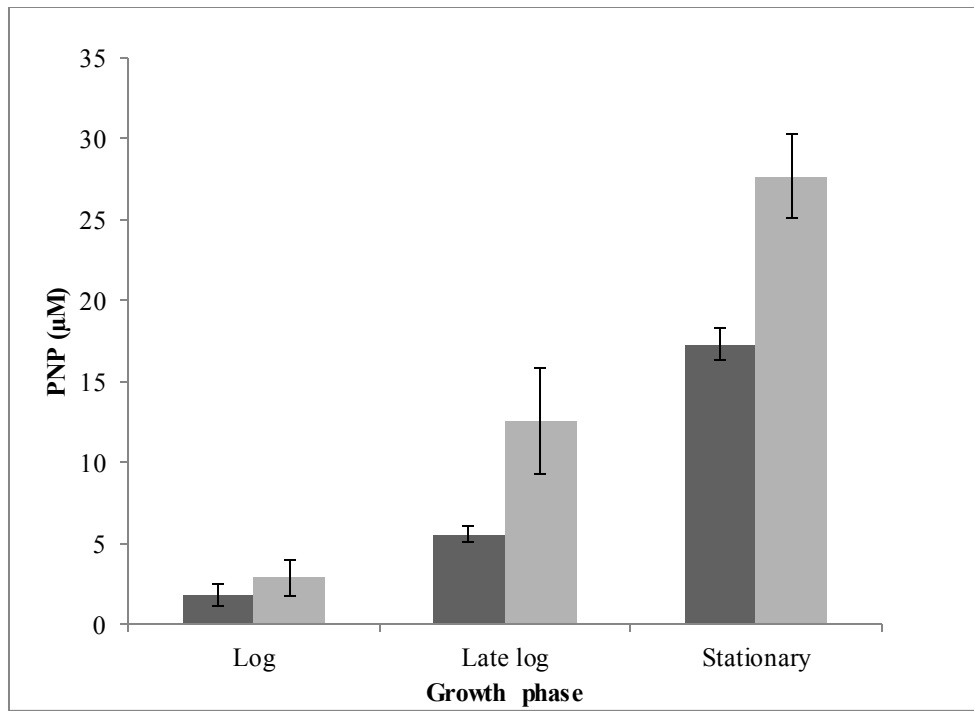
Figure 1

Figure 2

Figure 3

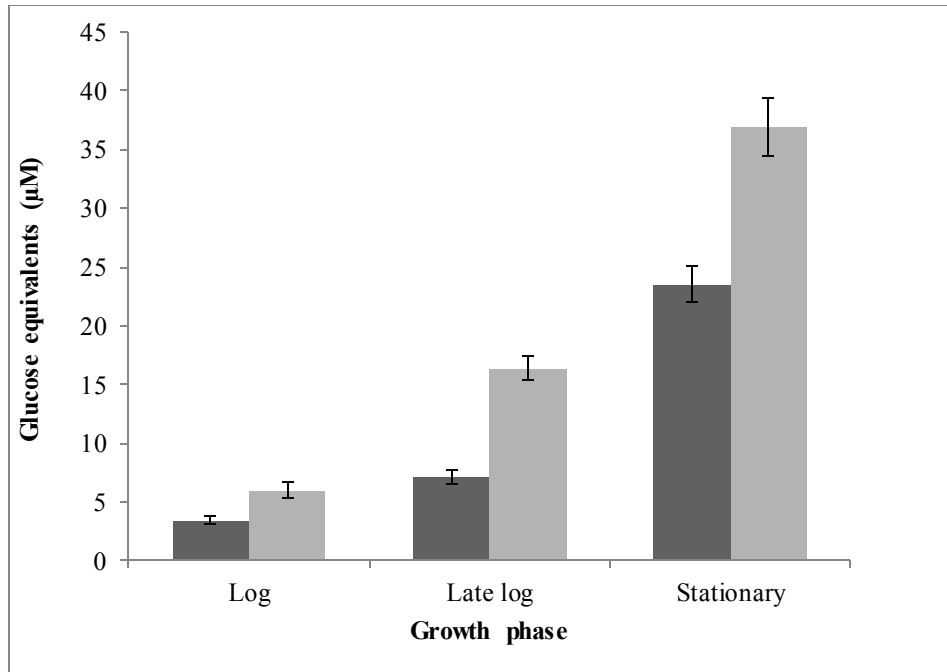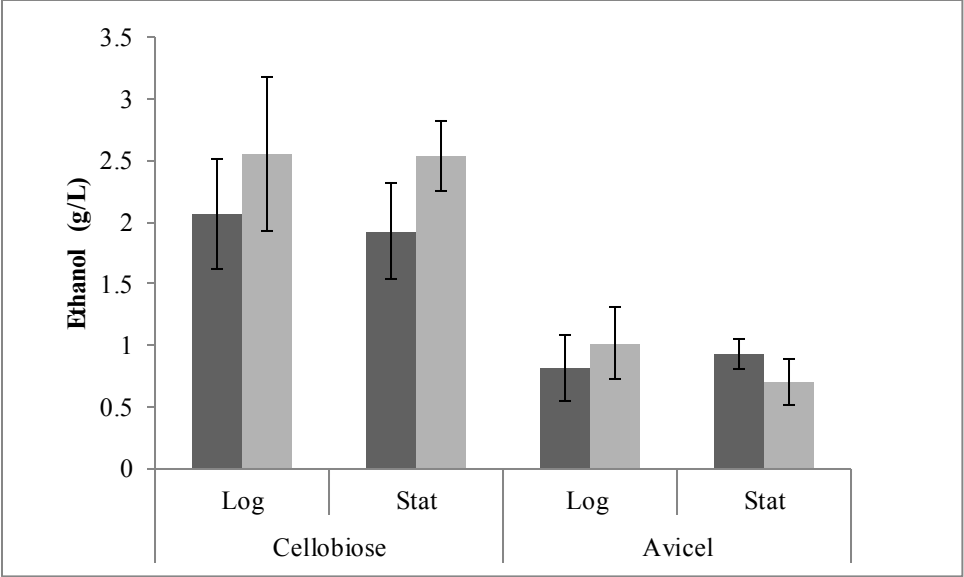**Figure 4**

Chapter 5: Development of a stable *Clostridium thermocellum* and *Thermoanaerobacterium saccharolyticum* co-culture for industrial production of bioethanol

In preparation

Authors: Miranda Maki, Lachlan Armstrong, Kam Tin Leung, Wensheng Qin

5.1 Abstract

The *Clostridium thermocellum* and *Thermoanaerobacterium saccharolyticum* co-culture has potential for application in the industrial production of biofuels. This co-culture has been favoured by researchers because of its potential to consolidate hydrolysis and fermentation steps in a process of consolidated bioprocessing (CBP) and potentially increase bioethanol titres in biofuel production by maximizing fermentation of 5- and 6-carbon sugars. However, there is little knowledge on the industrial application of this kind of co-culture including substrate conditions and the number of generations, all in conjunction with the effect on ethanol titres. The goal of this study was to develop a stable co-culture of *C. thermocellum* 27405 and *T. saccharolyticum* 31097 which can produce greater ethanol titres than mono-cultures in batch fermentation. Comparison of *C. thermocellum* and *T. saccharolyticum* growth in reducing sugar (1% (w/v) cellobiose and 0.5% (w/v) xylose) and polysaccharide (1% (w/v) Avicel and 0.5% (w/v) cellobiose) medium, showed that *T. saccharolyticum* could grow 2-fold faster in reducing sugar medium compared to *C. thermocellum*, whereas *C. thermocellum* grew to 2.3-fold greater turbidity in polysaccharide medium in mono-cultures. Correspondingly, co-culture batch fermentation trials revealed that both strains could only co-exist for one generation in reducing sugar medium, as confirmed by biomarker genes (*bgl*A and *xyl*B, respectively) detected by PCR, while in the consecutive generations only *T. saccharolyticum* was detected. In polysaccharide medium, both strains were detected for a continuous 4 generations in batch fermentation trials, using the same biomarker genes. After the fourth

generation, the co-culture requires re-establishing or further media optimization due to growth inhibition of strains, possibly a consequence of toxic by-products such as ethanol. Additionally, the ethanol titres also increased by 2.01-fold in the first and second generation compared to the mono-cultures. However, third and fourth generations did not have significantly different ethanol titres. Nonetheless, *C. thermocellum* and *T. saccharolyticum* co-culture has potential application if added during the hydrolysis stage of complex polysaccharides but not if added to simple sugars such as mono-, di-, or short oligo-saccharides of the fermentation stage.

5.2 Introduction

In the world today, pressures are mounting towards the development of an economically feasible, sustainable and „greener" fuel source. The bioconversion of lignocellulosic biomass into products such as bioethanol, offers potential towards a cleaner renewable fuel source; however, the current process lacks economic feasibility mainly due to costly pretreatment and rate-limiting hydrolysis (McMillan, 1997; Farrell *et al.*, 2006). Presently, bioconversion of lignocelluloses is a multi-step process; in particular, it requires the use of enzymes typically produced by *Trichoderma reesei* in a step called hydrolysis or saccharolysis, then in a separate step referred to as fermentation, yeast and/or bacteria are used to ferment 5- and 6-carbon sugars to ethanol.

The use of *Clostridium thermocellum* has been suggested by many researchers because of its ability to combine the hydrolysis and fermentation steps in a process referred to as consolidated bioprocessing (CBP) (Demain *et al.,* 2005; Maki *et al.*, 2009). *C. thermocellum* has one of the highest rates of hydrolysis for microcrystalline cellulose, comparable to *T. reesei*. Additionally, *C. thermocellum* is a Gram positive, anaerobic, thermophile, thus there would be no additional costs required for aeration, as well as less costs associated with cooling after pretreatment and reheating for ethanol evaporation (Demain *et al.,* 2005). However, there are some important limitations; despite the ability to hydrolyze

both hemicelluloses (5- and 6-carbon sugars) and celluloses (glucose monomers). *C. thermocellum* can only ferment 6-carbon sugars; thus, the 5-carbon sugars would fall to waste.

Co-culturing of *C. thermocellum* with a microorganism which can ferment 5- and 6-carbon sugars to ethanol has been readily suggested (Ng *et al.*, 1981; Mori 1990; Demain *et al.*, 2005). *C. thermocellum* has been shown to be amenable in growth with several closely related thermophilic anaerobic bacteria such as *Clostridium thermosaccharolyticum,* now classified as *Thermoanaerobacterium saccharolyticum*, (Venkateswaren and Demain, 1986; Saddler and Chan, 1985), *Clostridium thermohydrosulfuricum* (Ng *et al.*, 1981; Saddler and Chan, 1985; Germain *et al.*, 1986), *Thermoanaerobacter ethanolicus* (Wiegel and Ljungdahl, 1979) and *Thermoanaerobium brockii* (Lamed and Zeikus, 1980). Specifically, in 2005 Demain *et al.* proposed a model whereby *C. thermocellum* would be amenable to grow with *Thermoanaerobacterium saccharolyticum*, an anaerobic thermophilic clostridia which has the ability to ferment 5- and 6-carbon sugars to ethanol. Thus, *C. thermocellum* would hydrolyze the cellulose and hemicelluloses but only ferment 6-carbon sugar products, while simultaneously *T. saccharolyticum* has the ability to ferment 5- and 6-carbon sugar products (Demain *et al.*, 2005).

Bacteria co-exist in the environment living in concert with other microorganisms, sometimes in competition and while others maintain symbiotic relationships. Further still, some bacterial species may inhibit growth and others still promote growth. Thus, bacterial consortia have often been described as complex networks where the co-existence of all the microorganisms in a stable co-culture depends on a variety of factors including growth requirements such as temperature, media (carbon source) and atmosphere of each strain involved, not to mention the interactions of each strain in the community as previously described (Kato *et al.,* 2005; Kato *et al.* 2008).

The use of co-cultures for the industrial bioconversion of lignocelluloses is premature; however it represents a plausible means in the use of *C. thermocellum* for CBP. Another possibility to improve ethanol titres through the fermentation of 5-carbon sugars could include metabolic engineering; however,

there are limited advances in the genetic manipulation of this anaerobic thermophile with a strict restriction endonuclease system (Klapatch *et al*, 1996). By gaining more knowledge, including the generation times and inoculation times, one could potentially develop a stable co-culture with *T. saccharolyticum* to improve the use of *C. thermocellum* for CBP.

In this study, we strive to further understand and develop a more stable co-culture of *C. thermocellum* and xylose-fermenting *T. saccharolyticum* to improve ethanol titres and application for CBP in the conversion of lignocellulosic biomass to bioethanol.

5.3 Materials and Methods

5.3.1. Media, strains and cultivation conditions

The strains *Clostridium thermocellum* (ATCC 27405) and *Thermoanaerobacterium saccharolyticum* (ATCC 31907) were purchased from the American Type Culture Collection through Cedarlane Labs, Canada. *C. thermocellum* mono-culture cells were grown at 57°C in chemically defined *Clostridium thermocellum* Medium (ATCC medium 1191) with a mineral elixir, reducing solution and vitamin solution according to the ATCC recipe and *T. saccharolyticum* mono-cultures were grown in a rich chemically undefined beef liver medium for anaerobes (ATCC medium 38), also at 57°C. Two co-culture media: one reducing sugar and one polysaccharide, were developed here using *Clostridium thermocellum* medium as the base. Reducing sugar medium contained 1% (w/v) cellobiose and 0.5% (w/v) xylose; whereas, the polysaccharide medium contained 1% (w/v) Avicel and 0.5% (w/v) beechwood xylan, as the sole carbon sources. All of the work in this study using *C. thermocellum* and *T. saccharolyticum* cells was done inside a Coy Anaerobic Chamber (Coy Laboratories, USA) under 5% hydrogen, 95% nitrogen mixed atmosphere, except when reading the cell optical densities.

5.3.2. Growth curve analysis on reducing sugar and polysaccharide media

Cells of *C. thermocellum* and *T. saccharolyticum* were individually inoculated under anaerobic conditions to 6 ml of reducing sugar and polysaccharide co-culture medium in 200 μl aliquots from 6 ml of 48 h cultures in their respective growth mediums. After 48 and 72 h of growth for reducing sugar and 72 h of growth for polysaccharide medium, respectively, they were subcultured one additional time, as previously described. *C. thermocellum* was grown for 72 h, while *T. saccharolyticum* for 48 h in reducing sugar medium due to the observed growth rate difference in them, while both had slower obseeved growth rates in polysaccharide medium.

 Then, after 48 and 72 h or 72 h growth 500 μl of each strain were then inoculated to 40 ml of reducing sugar and polysaccharide co-culture medium in triplicate, respectively, creating an initial cell density of approximately 0.15 O.D.$_{600nm}$. Triplicate samples of 300 μl were taken from each vial at various intervals for reducing sugar and polysaccharide medium: 0, 12, 15, 18, 21, 24, 27, 30, 33, 36, 39, 41, 44, 48, 52, 46, 60, 64 h; and 0, 24, 30, 35,40, 45, 50, 55, 60, 65 and 70 h, respectively. Each sample was loaded in triplicate to a 96-well microtitre plate and the absorbance at 600 nm was read using an xMark microplate spectrophotometer (Bio-Rad Laboratories, Canada).

5.3.3. Co-culture and mono-culture batch culture trials

As previously mentioned, cells of *C. thermocellum* and *T. saccharolyticum* were pre-grown twice in reducing sugar and polysaccharide co-culture media before final growth in 40 ml of appropriate co-culture media to ~0.6 O.D.$_{600nm}$. To create co-cultures, the growth curves were used to determine log phase inoculation times for each strain (*C. thermocellum* and *T.saccharolyticum*) from reducing sugar and polysaccharide co-culture media. For reducing sugar media, due to the accelerated growth rate of *T. saccharolyticum* compared to *C. thermocellum* cells were inoculated in triplicate to 50 ml of reducing sugar media in a 2:1 ratio for *C. thermocellum* and *T. Saccharolyticum*, respectively, based on volume using cell cultures of similar O.D.$_{600nm}$~0.6. However, to develop co-cultures in polysaccharide medium,

T. saccharolyticum and *C. thermocellum* were inoculated in triplicate in a 1:1 ratio, respectively, using similar O.D.$_{600nm}$ cultures. All mono-culture and co-cultures on reducing sugar and polysaccharide media were allowed to grow to mid stationary phase (~0.8 O.D.$_{600nm}$) at 57°C, to constitute one full growth culture based on growth response curve analysis. Consecutive cultures, subcultured from the previous, were inoculated in triplicate from mid stationary phase by transferring 500 μl of each mono- and co-cultures to 50 ml of fresh reducing sugar and polysaccharide media.

5.3.4. Detection of C. thermocellum and T. saccharolyticum persistence using biomarkers

The presence of each strain: *C. thermocellum* and *T. saccharolyticum*, was detected in co-culture using uniquely designed biomarkers. For each strain in triplicate experiments, at each continuous subculture, (reducing sugar media subcultures 1-3, and polysaccharide media subcultures 1-5), 3 ml of mid stationary phase cells were collected for extraction of genomic DNA using the UltraClean Microbial DNA Isolation Kit (MediCorp, Canada), following the instructions provided by the supplier and using the troubleshooting option for „difficult to lyse cells". The β-glucosidase A gene (*bgl*A) was selected as a biomarker for the presence of *C. thermocellum*. Forward and reverse primers were used to amplify a ~500 bp region of *bgl*A (primer sequences refer to Table 1). Similarly, the β-xylosidase B gene (*xyl*B) was chosen to be the biomarker in detection of *T. saccharolyticum*. Forward and reverse primers were designed to amplify a ~700 bp region within *xyl*B (for primer sequences refer to Table 1). The PCR reaction mixture contained ~10 ng of *C. thermocellum/T. saccharolyticum* genomic DNA, 10 pmol of both appropriate forward and reverse primers, 10x Taq buffer with 500 mM KCl, 25 mmol l^{-1} MgCl$_2$, 0.2 mmol deoxynucleoside triphosphate, and 5 U DNA polymerase per 50 μl reaction. The PCR program was as follows: primary denaturation 3 minutes at 95°C, followed by 35 amplification cycles consisting of denaturing at 95°C for 30 seconds, annealing for 1 minute at 54°C, and extension at 72°C for 30 seconds, upon completion of 35 amplification cycles a final extension step was done at 72°C for 10 minutes.

5.3.5. Analytical methods for ethanol

Supernatant samples containing ethanol samples were collected from late stationary phase (~1.5 O.D.$_{600nm}$, 96h growth) batch culture trials of mono-cultures for *C. thermocellum* and *T. saccharolyticum* and co-culture batch culture trials from polysaccharide medium containing Avicel and xylan as previously mentioned, by aliquoting 1.5 ml of culture supernatant into 1.5 ml Eppendorf tubes. All samples were stored at -20°C until analysis. The samples were analyzed using an Agilent 6850 Gas Chromatograph fitted with a Carbowax column (30 m x 0.32 mm, film thickness 0.25 μm) and flame ionization detector. In preparation for analysis, the samples were thawed at room temperature and 1 ml of each sample was transferred to a fresh 1.5 ml Eppendorf tube and centrifuged at 17,000 *g* for 5 min. Prior to injection, each sample was spiked with 100 μl *n*-butanol, which acted as an internal standard. Injection volume was 1μl and the inlet was run splitless. Nitrogen was used as a carrier gas with a flow rate of 1.5 ml/min and the run time was 5 minutes/sample. The injection port temperature was set at 250°C, the column temperature was isothermally set at 75°C, and the detector temperature was 300°C. Standards were prepared the day of analysis using anhydrous ethanol and they were also spiked with 100 μl *n*-butanol per 1 ml standard.

5.4 Results

5.4.1. Growth response curves in reducing sugar and polysaccharide media

Cell counts were difficult to obtain due to the lower number of cells producing colonies in or on solid agar. Confocal scanning laser microscopy (CFLSM) was used to make direct cell counts using live and dead staining technique, however higher variation resulted in lower correlation with O.D.$_{600nm}$ spectrophotometer readings. Cell counts were not vital for the production of a stable co-culture. Growth curves were developed using spectrophotometry readings of turbidity at O.D.$_{600nm}$. In Figure 1, for reducing sugar medium containing cellobiose and xylose, it was found that mono-cultures of *C. thermocellum* and *T. saccharolyticum* were able to enter log phases of growth at approximately 30 and 15 h growth, respectively. Thus, *C. thermocellum* took twice as long to enter log phase of growth. However,

upon reaching stationary phases of growth at 56 h *C. thermocellum* and *T. saccharolyticum* had nearly equal turbidities O.D.$_{600nm}$ of 1.05 and 1.09, respectively.

However, in Figure 2 polysaccharide medium, *C. thermocellum* and *T. saccharolyticum* entered log phases of growth at approximately equal time of 24 h. However, upon reaching stationary phases at 60 and 50 h, respectively, turbidity readings at O.D.$_{600nm}$ were 1.441 and 0.632, respectively. Thus, *C. thermocellum* could reach a 2.3-fold greater turbidity than *T. saccharolyticum* on polysaccharide medium at stationary phases of growth.

5.4.2. Determining co-culture stability with biomarkers

PCR amplification of biomarker genes *bgl*A and *xyl*B were used to determine the presence of *C. thermocellum* and *T. saccharolyticum* in co-culture, respectively. As shown in Figure 4, for the control mono-cultures of *C. thermocellum* and *T. saccharolyticum*, PCR for each specific biomarker (*bgl*A and *xyl*B, respectively) only produced a single band of ~500 bp and 700 bp, respectively in the species it was designed for, making them excellent biomarker candidates. Batch fermentation co-culture trials in reducing sugar media, shown in Figure 3, show that *C. thermocellum and T.saccharolyticum* were present upon completion of first generations; however, in the second and consecutive generations only *xyl*B was detected, as was the case for subsequent generations not shown here. This indicated that *T. saccharolyticum* was the primary strain present. On the contrary, in polysaccharide media, both biomarker genes were detected continuously for generations 1 to 4 (Figure 4). The PCR biomarkers were not detected in the 5th generation for all triplicate fermentation trials.

5.4.3. Ethanol titres from mono- and co-cultures in polysaccharide media

Ethanol titres (g l^{-1}) (Figure 5) were measured after 96 h growth upon reaching late stationary phase (~1.5 O.D.$_{600nm}$) for *C. thermcocellum* (CT) and *T. saccharolyticum* (TS) co-cultures of PCR detectable generations 1 through 4 in batch fermentation trials. Similarly, ethanol titres of mono-cultures for CT and

TS were also measured after stationary phase growth in polysaccharide medium. It was found that generations 1 through 4 had significantly greater ethanol titres than TS mono-cultures, $p < 0.05$ (Student"s t-test), having an average 4.7-fold greater ethanol production than TS mono-cultures. However, it was found that only generations 1 and 2 had significantly greater ethanol titres than CT mono-cultures, $p < 0.05$ (Student"s t-test), with an average 2.1 fold greater ethanol titres. Generations 3 and 4 did not display significantly different ethanol titres than CT and TS mono-cultures.

5.5 Discussion

The use of *C. thermocellum* and *T. saccharolyticum* in co-culture to improve ethanol titres requires further knowledge for industrial application and cost reduction. Currently, there is a lack of knowledge on the number of generations and the overall effect on ethanol titres for the most cost effective industrial use.

A variety of xylanase genes including β-xylosidases also exhibiting cellobiohyrdrolase activity (Lee and Zeikus, 1993; Lorenz and Weigel, 1997), endoxylanases (Lee *et al.*, 1993; Lee and Zeikus, 1993), and alpha-D-glucuronidases (Bronnenmeir *et al.*, 1995) have been found and characterized in the extracellular production from *T. saccharolyticum* strains, however no endo- or exo-cellulases have been detected in this bacterial species indicating that the metabolism is different which may eliminate competition for substrate; this made it a good candidate for co-culture compatibility with *C. thermocellum*.

In contrast, *C. thermocellum* is well known for its ability to degrade crystalline cellulose and its ability to produce free cellulases as well as those in multienzyme complexes referred to as cellulosomes (Lamed and Bayer, 1988). It has also been shown to produce several xylanases in cellulosome complexes (Gold and Martin, 2007). In co-culture with *T. saccharolyticum*, it has been suggested as the „work-horse" producing the majority of enzymes for effective degradation (Demain *et al.*, 2005).

The enzymes produced and thus the metabolic capabilities of each strain lend knowledge into our development of a stable co-culture. Therefore, the substrates used for media composition have a direct result on the sustainability of the co-culture as shown by our comparison of reducing sugar and polysaccharide media. *T. saccharolyticum* could grow more rapidly in reducing sugar media containing simple sugars such as xylose and cellobiose. No matter the inoculation ratio, we found that *T. saccharolyticum* always outgrew *C. thermocellum* by the second generation. We presume that the detection of *C. thermocellum* in the first generation was due to the higher inoculation densities required and not necessarily due to proliferation. Competition for substrates played a substantial role here which is referred to as antagonism between the species. Competition like this has been shown in the growth of bacteria in the gut of rats (Guiot, 1982) and in a variety of rumenal bacteria from ruminants (Russell and Baldwin, 1982). Thus, the use of a *C. thermocellum* and *T. saccharolyticum* co-culture for application of fermentation of simple sugars would be impractical for industrial production of biofuels.

However, our results in polysaccharide medium, that is medium containing sugars with greater depolymerization such as xylan and Avicel, showed that at a 1:1 inoculation ratio of *C. thermocellum* and *T. saccharolyticum* could allow for their co-survival for 4 continuous generations in batch fermentation. This would suggest a synergism between each species (Kato *et al.*, 2005). Moreover, this co-stability also rendered greater ethanol titres when compared to mono-culture batch fermentation trials with each strain. Similarly, in 2010 Fang observed increased ethanol titres when co-culturing *C. thermocellum* LQRI and *T. pseudoethanolicus* X514 or *T. ethanolicus* 39E when grown on the cellulosic substrate Solka Floc, compared to mono-cultures (Fang 2010).

In polysaccharide medium, we saw significant growth (increase in turbidity) of *C. thermocellum* after 72 h. However, in growth curve analysis of *T. saccharolyticum* we observed little to no change in growth (turbidity) after 72 h. This is a reflection on the metabolic activity of each strain. As previously mentioned *C. thermocellum* possessed the enzymes for efficient hydrolysis of Avicel and also contributed to the degradation of xylan, whereas *T. saccharolyticum* could not utilize these substrates without the

147

presence of *C. thermocellum* because it lacks the enzymatic capabilities of converting them to small poly- and oligo-saccharides which can be taken up by the cell and further metabolized by fermentation into ethanol. Thus, we suspect that after *C. thermocellum* initiates enzymatic degradation of the Avicel and xylan, *T. saccharolyticum* begins to grow thereby contributing to greater ethanol titres.

However, the co-culture cannot be transferred to a 5th generation successfully in all triplicate experiments. We suggest that toxic inhibitory end-products such as ethanol and organic acids such as acetate and lactate, cause stress which disrupts cell growth rendering the cells non-culturable. Studies have mentioned time and again that ethanol, as well as organic acids have a toxic inhibitory effect on the limiting growth of these anaerobic ethanogenic bacteria (Lynd, 1989; Rani and Seenayya, 1999; Burdette *et al.*, 2002; Zeng *et al.*, 2004).

Additionally, the apparent stability of our co-culture did not relay to stable ethanol titres for all four generations. It was observed that in the first two generations of co-culture there were greater ethanol titres than generations three and four, which were not significantly different from *C. thermocellum* mono-cultures. Large variation was also seen in the detection of ethanol, particularly in the fourth generation. The reduced ethanol production in third and fourth generation ethanol titres suggests changes in the dynamics of the co-culture. The changes may be attributed to a variety of issues such as end-product toxicity, shifts in the population densities over time or adaptive changes in the interactions between species, such as metabolism. However, all generations had significantly greater ethanol titres than *T. saccharolyticum* mono-cultures, which was not surprising due to the limited turbidity observed in comparison to *C. thermocellum* in polysaccharide medium growth curves.

Nonetheless, our results show that *C. thermocellum* and *T. saccharolyticum* co-culture can represent a feasible means to decompose complex polysaccharides such as hemicelluloses and celluloses and ultimately convert them to bioethanol in a single process (CBP). With recent advancements in the development of strains with greater ethanol tolerance (Shao *et al.,* 2011), higher cellulase degrading abilities and knockouts which do not produce acidic by-products such as acetate (Tripathi, *et al.*, 2010) or

148

lactate (Desai *et al.*, 2004), applied with our co-culture information can be used to improve the industrial production of biofuels from lignocellulosic biomass, making it economically more feasible.

Acknowledgements

References

Burdette, D.S., Jung, S.H., Shen, G.J., Hollingsworth, R.I. and Zeikus, J.G. (2002) Physiological function of alcohol dehydrogenases and long-chain (C-30) fatty acids in alcohol tolerance of *Thermoanaerobacter ethanolicus*. *Appl Environ Microbiol* **68**, 1914–1918.

Bronnenmeier, K., Meissner, H., Stocker, S. and Staudenbauer, W.L. (1995) alpha-D-glucuronidases from the xylanolytic thermophiles Clostridium stercorarium and Thermoanaerobacterium saccharolyticum. *Microbiology* **141**, 2033-2040.

Demain, A.L., Newcomb, M. and Wu, J.H.D. (2005) Cellulase, Clostridia, and Ethanol. *Microbiol Mole Biol Rev* **69**, 124–154.

Desai, S.G., Guerinot, M.L. and Lynd, L.R. (2004) Cloning of L-lactate dehydrogenase and elimination of lactic acid production via gene knockout in *Thermoanaerobacterium saccharolyticum* JW/SL-YS485. *Appl Microbiol Biotechnol* 65, 600–605.

Fang, Z.G. (2010) Enhanced role of the co-culture of thermophilic anaerobic bacteria on cellulosic ethanol. *Huan Jing Ke Xue* **31**, 1059-65.

Farrell, A.E., Plevin, R.J., Turner, B.T., Jones, A.D., O"Hare, M. and Kammen, D.M. (2006). Ethanol can contribute to energy and environmental goals. *Science* **311**, 506–508.

Germain, P., Toukourou, F. and Donaduzzi, L. (1986) Ethanol production by anaerobic thermophilic bacteria: regulation of lactate dehydrogenase activity in Clostridium thermohydrosulfuricum. *Appl Microbiol Biotechnol* **24**, 300–305.

Guiot, H.F. (1982) Role of competition for substrate in bacterial antagonism in the gut. *Infect Immun* **38**, 887-892.

Kato, S., Haruta, S., Cui, Z.J., Ishii, M. and Igarashi, Y. (2008) Network relationships of bacteria in a stable mixed culture. *Microb Ecol* **56**, 403-311.

Kato, S., Haruta, S., Cui, Z.J., Ishii, M. and Igarashi, Y. (2005) Stable coexistence of five bacterial strains as a cellulose-degrading community. *Appl Environ Microbiol* **71**, 7099-7106.

Klapatch, T.R., Demain, A.L., Lynd, L.R. Restriction endonuclease activity in Clostridium thermocellum and Clostridium thermosaccharolyticum. *Appl Microbiol Biotechnol* **45**, 127-131.

Lamed, R. and Zeikus, JG.(1980) Ethanol production by thermophilic bacteria: relationship between fermentation product yields of and catabolic activities in Clostridium thermocellum and Thermoanaerobium brockii. *J Bacteriol* **144**, 569-578.

Lamed, R., and Bayer, E. A. (1988) The cellulosome of *Clostridium thermocellum. Adv. Appl. Microbiol.* **33,** 1–46

Lee, Y.E. and Zeikus J.G. (1993) Genetic organization, sequence and biochemical characterization of recombinant beta-xylosidase from Thermoanaerobacterium saccharolyticum strain B6A-RI. *J Gen Microbiol* **139,** 1235-1243.

Lee, Y.E., Lowe, S.E., Zeikus, J.G. (1993) Gene cloning, sequencing, and biochemical characterization of endoxylanase from Thermoanaerobacterium saccharolyticum B6A-RI. *Appl Environ Microbiol* 59, 3134-3137.

Lorenz, W.W., Wiegel, J. (1997) Isolation, analysis, and expression of two genes from Thermoanaerobacterium sp. strain JW/SL YS485: a beta-xylosidase and a novel acetyl xylan esterase with cephalosporin C deacetylase activity. *J Bacteriol* **179,** 5436-41.

Lynd, L.R. (1989) Production of ethanol from lignocellulosic materials using thermophilic bacteria. Critical evaluation of potential and review. *Adv Biochem Engin/Biotechnol* **38**, 1-52.

Maki, M., Leung, K.T. and Qin, W. (2009) The prospects of cellulase-producing bacteria for the bioconversion of lignocellulosic biomass. *Int J Biol Sci* **5**, 500-516.

McMillan, J.D., (1997) Bioethanol production: status and prospects. *Renew Energy* **10**, 295–302.

Mori, Y. (1990) Characterization of a Symbiotic Coculture of *Clostridium thermohydrosulfuricum* YM3 and *Clostridium thermocellum* YM4. *Appl Environ Microbiol* **56**, 37-42.

Ng, T.K., Ben-Bassat, A. and Zeikus, J.G. (1981) Ethanol Production by Thermophilic Bacteria: Fermentation of Cellulosic Substrates by Co-cultures of Clostridium thermocellum and Clostridium thermohydrosulfuricum. *Appl Environ Microbiol* **41,** 1337-1343.

Rani, K.S. and Seenayya, G. (1999) High ethanol tolerance of new isolates of *Clostridium thermocellum* strains SS21 and SS22. *World J Microbiol Biotechnol* **15**, 173–178.

Russell, J.B. and Baldwin, R.L. (1979) Comparison of Substrate Affinities Among Several Rumen Bacteria: a Possible Determinant of Rumen Bacterial Competition. *Appl Environ Microbiol* **37,** 531.

Saddler, J.N. and Chan, M.K.H. (1985) Conversion of pretreated lignocellulosic substrates to ethanol by *Clostridium thermocellum* in mono- and coculture with *Clostridium thermosaccharolyticum* and *Clostridium thermohydrosulfuricum. Can J Microbiol* **30**, 212–220.

Shao, X., Raman, B., Zhu, M., Mielenz, J.R., Brown, S.D., Guss, A.M. and Lynd, L.R. (2011) Mutant selection and phenotypic and genetic characterization of ethanol-tolerant strains of Clostridium thermocellum. *Appl Microbiol Biotechnol* **92**, 641-52.

Tripathi, S.A., Olson, D.G., Argyros, D.A., Miller, B.B., Barrett, T.F., Murphy, D.M., McCool, J.D., Warner, A.K., Rajgarhia, V.B., Lynd, L.R., Hogsett, D.A. and Caiazza, N.C. (2010) Development of *pyrF*-Based Genetic System for Targeted Gene Deletion in *Clostridium thermocellum* and Creation of a *pta* Mutant. *Appl Environ Microbiol* 76, 6591-6599.

Venkateswaren, S. and Demain, A.L. (1986) The Clostridium thermocellum-Clostridium thermosaccharolyticum ethanol production process: nutritional studies and scale-down. *Chem Eng Commun* **45**, 53-60.

Weigel, J. and Ljungdahl, L.G. (1970) Ethanol as fermentation product of extreme thermophilic, anaerobic bacteria. In: H. Dellweg H, ed. In Klassische Prozesse mit Neun Aussichten. Berlin, germany: Verlag Versuchs und Lehranstalt fuer Spiritusfabrikation and Fermentation Technologie im insituut fuer Gaerungsgewerbe und Biotechnologie.117-127.

Zeng, A-P., Ross, A., Biebl, H., Tag, C., Günzel, B. and Deckwer, W-D. (2004) Multiple product inhibition and growth modeling of *clostridium butyricum* and *klebsiella pneumoniae* in glycerol fermentation. *Biotechnol Bioengin* 44, 902-911.

Table 1. Primers designed for the application of biomarker genes and expected product size for i) *Clostridium thermocellum* (*bglA*), and ii) *Thermoanaerobacterim saccharolyticum* (*xynB*).

Gene	Forward (5"-3")	Reverse (5"-3")	Product (bp)
bglA	ATCTGGACTCGGAGGTGTAT	TTGTGCCATACCAACCATG	538
xynB	ATACAGGTACGCCAAGAGGA	AGTAGTCAGCACCACCGCAT	684

Figure Legend

Figure 1. Growth response curve of *C. thermocellum* (●) and *T. saccharolyticum* (▲) in reducing sugar medium, measure by turbidity at $O.D._{600nm}$.

Figure 2. Growth response curve of *C. thermocellum* (●) and *T. saccharolyticum* (▲) in polysaccharide medium, measure by turbidity at $O.D._{600nm}$.

Figure 3. PCR detection of *C. thermocellum* and *T. saccharolyticum* in reducing sugar medium batch fermentations using biomarkers *bgl*A and *xyl*B, respectively. Lanes: 1.1kb DNA ladder, 2-3. Generation 1 and 4-5. Generation 2.

Figure 4. PCR detection of *C. thermocellum* and *T. saccharolyticum* in polysaccharide medium using biomarkers *bgl*A and *xyl*B, respectively. Lanes: 1.1kb DNA ladder, 2-3. Generation 1, 4-5. Generation 2, 6-7. Generation 3, 8-9. Generation 4.

Figure 5. The ethanol titres (g l^{-1}) of *C. thermocellum* and *T.saccharolyticum* co-culture generations 1 through 4, and mono-cultures of *C. thermocellum* and *T. saccharolyticum* all in polysaccharide medium batach fermentation trials.
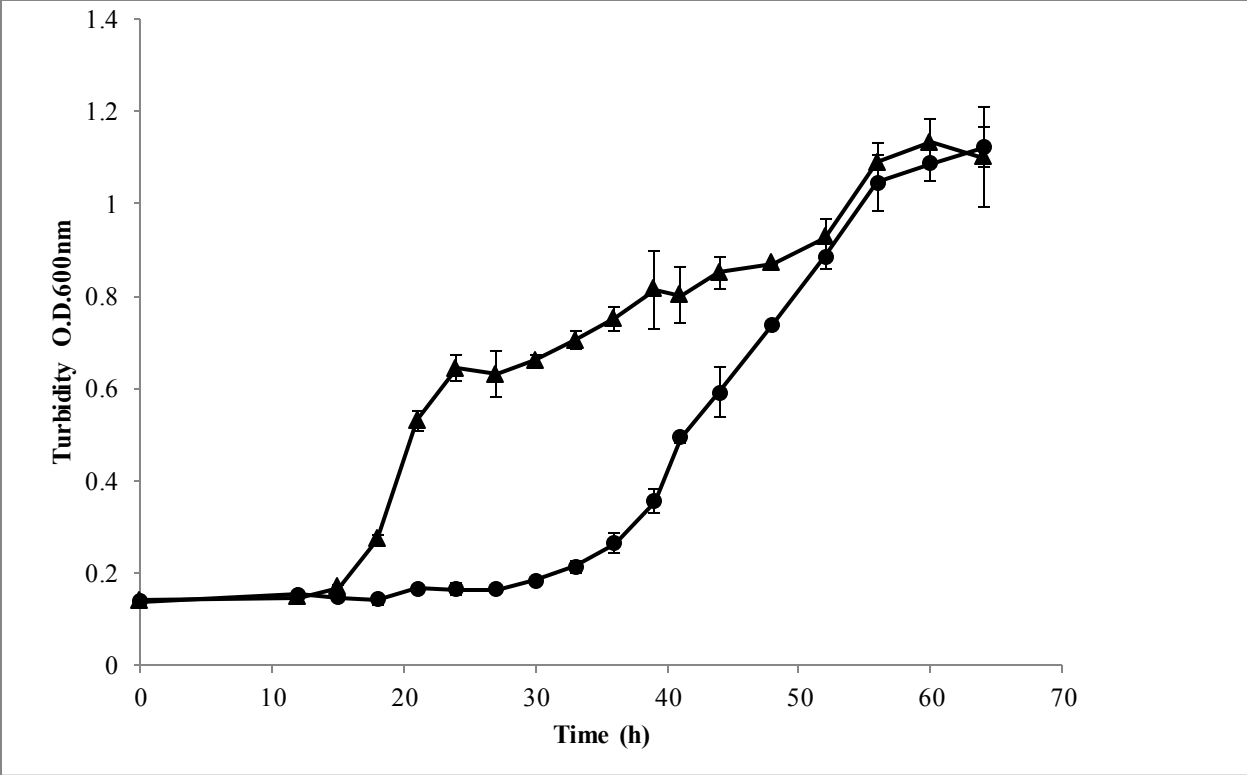
154

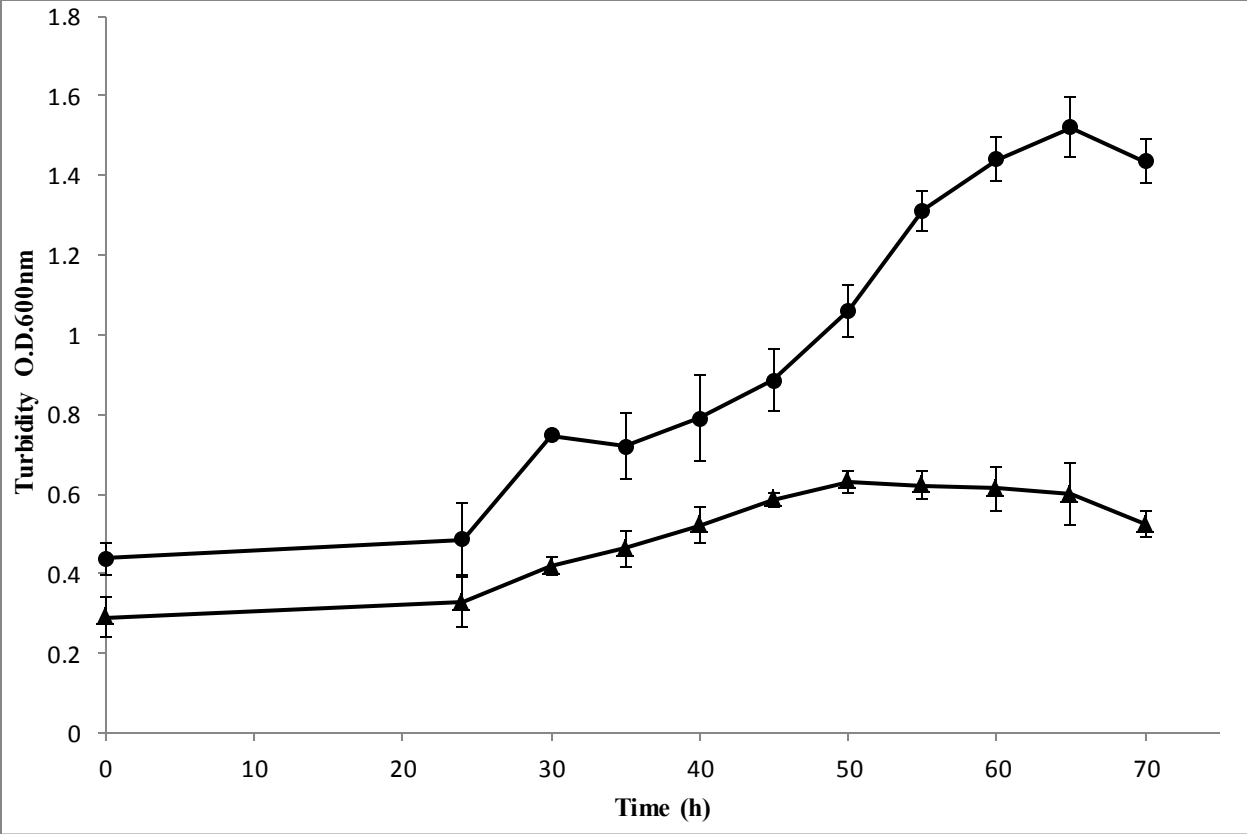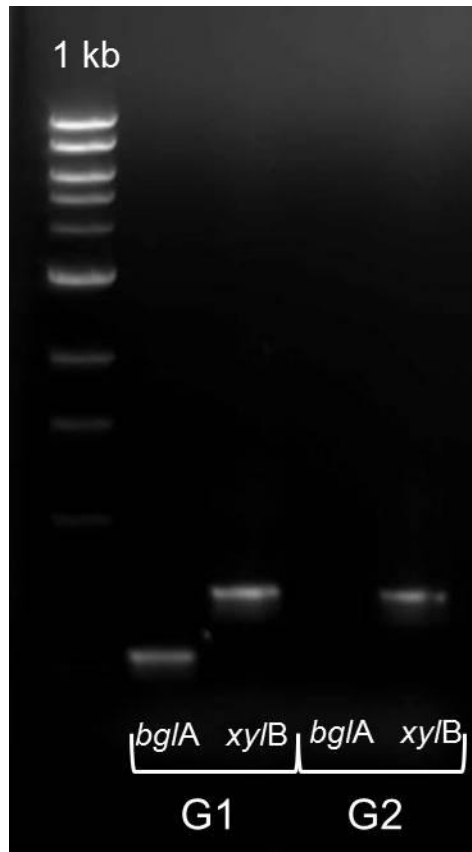**Figure 1**

Figure 2

Figure 3

Figure 4

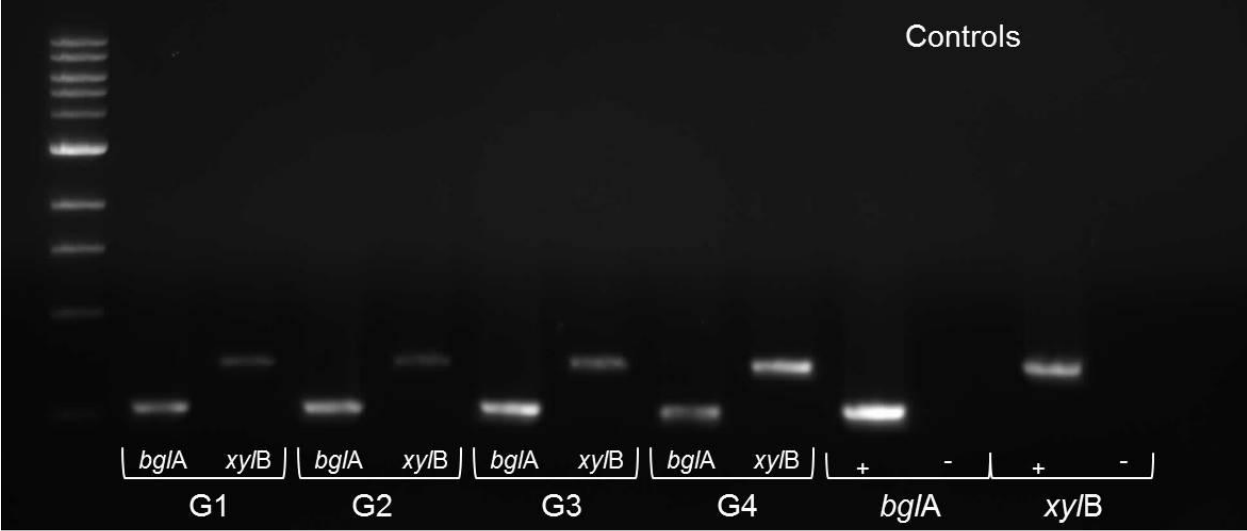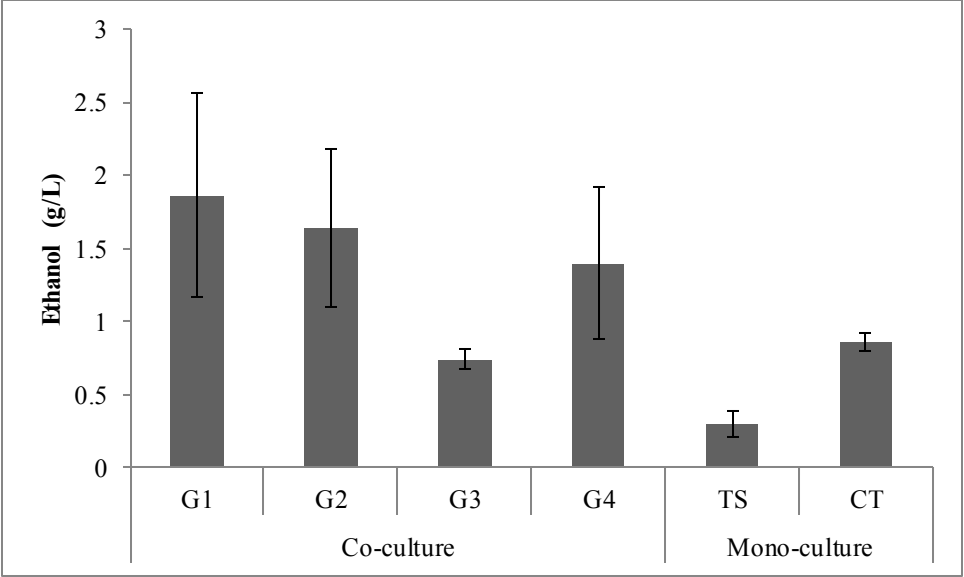**Figure 5**

Chapter 6: General discussion and future directions

Global climate change, uncertain sources of petroleum and rising costs of fuel have sparked a worldwide search for „greener" energy replacements (Schneider, 1989). Lignocellulosic biomass („plant biomass") is the least controversial, most abundant source of organic biomass which has been rapidly gaining attention for the bioconversion by microorganisms such as (fungi, bacteria and yeast) to products such as bioethanol and biobutanol, not to mention other value-added by-products such as organic acids. However, there are currently several challenges facing its economic development. For one, the conversion of lignocellulosic biomass to products such as bioethanol is a costly process which requires pretreatment with high temperature and often the addition of acids or bases to to increase surface area and porosity, remove lignin and hemicelluloses and disrupt the crystallinity of cellulose (Wyman *et al.*, 2005). Further still, after pretreatment, cooling and neutralization may be required before the addition of enzymes in the hydrolysis step. Then in a consecutive step, there is the addition of subsequent microorganisms (i.e. *Saccharomyces cerevisiae*) to ferment short polysaccharides or monosaccharides to bioethanol (Philippidis *et al.,* 1993; Lynd *et al.*, 2005). Finally, the system must be heated and distilled to collect the ethanol. This process can be costly; as well as, damaging and toxic to the enzymes and microorganisms used.

Furthmore, lignocellulosic biomass is composed of mainly cellulose (glucose monomers), then hemicellulose (5- and 6-carbon sugars) and least of all lignin (complex polyphenol). The content of each varies widely between plant parts and plant species, requiring different pretreatments and a large variety of enzymes to efficiently hydrolyze. Thus, there are no single microorganisms which can produce all the required enzymes to efficiently hydrolyze lignocellulosic biomass. Other challenges include, end-product inhibition of enzymes, microorganisms producing enzymes or performing fermentation.

All of the work presented here in this thesis, approaches these challenges in the current production of biofuels from different perspectives with a main focus on bacteria. There were several

reasons for this choice, namely bacteria can be easily cultured, can be found inhabiting unlimited environments and can survive in extreme environmental stresses. These attributes lend to the potential exploitation of hardier enzymes for the industrial conversion of lignocelluloses to biofuels. Also, these attributes could allow for the development of whole cell systems which can work synergistically to consolidate processes such as hydrolysis and fermentation in a single step, known as consolidated bioprocessing (CBP) (Lynd *et al.*, 2005).

The research presented in this thesis led to the discovery of new cellulase- and lignocellulase-producing bacteria with potential in future studies for the characterizarion and exploitatition of their enzymes in the hydrolysis of lignocellulosic biomass. Additionally, this work lends knowledge to the future study and use of *C. thermocellum* in the simultaneous hydrolysis and fermentation of cellulose to ethanol.

Firstly, my work focused on the isolation and characterization of efficient cellulase-producing bacteria. With this work I was able to develop an efficient and economical method for screening large numbers of bacteria from different environmental and commercial sources to find the most efficient and potentially unique cellulase-producing bacteria which have potential for downstream application in the industrial production of biofuels. Two isolates, E2 and E4, both *Paenibacillus* species were found in this study to have the greatest total cellulase activity, representing activities towards soluble (carboxymethyl cellulose) and insoluble/crystalline cellulose (Whatman no.1 filter paper). In biotechnological applications such as the production of biofuels from lignocellulosic biomasses looking for new industrial enzymes or bacteria producing enzymes, traditional microbiological isolation techniques are still important. There are several recent studies on the isolation and characterization of cellulases from newly isolated bacteria (Fu *et* al., 2010; George *et al.*, 2010; Okeke and Lu, 2010; Yang *et al.*, 2010). This is because the hydrolysis stage remains a rate-limiting step due to the efficiency of enzymes, such as cellulases (Rivers and Emert, 1988).

The total 18 cellulase-producing bacteria isolated in this study represent a foundation for the exploitation of unique cellulases which may be more efficient or resilient in the industrial environment. Future work is required to characterize those isolates exhibiting greater cellulase activities. This includes looking at the effects of pH, temperature and inducers, on the optimal conditions for increased expression of cellulases. To do such work, requires a smaller sample size (focus on one or two of the greatest cellulase producing strains such as E2 and E4). Moreover, if a future study focuses on just one or two strains, there is a greater potential to identify whether the enzymes are solely secreted in the supernatant, expressed internally or associated with the cell surfaces. Cell associated cellulases have been previously identified in *Paenibacillus* species (Pastor *et al.*, 2001; Waeonukul *et al.*, 2009; Pason *et al.*, 2010). Identification of the source of enzymes, (i.e. supernatant or cell associated), is valuable information which can be used not only for the industrial production of such enzymes but also in lab-scale purification for further biochemical characterization.

In my second study, I used our previously established microbiological techniques in the isolation and characterization of lignocellulase-producing bacteria. That is, to identify bacteria which have a greater effect on the overall degradation of lignocellulosic biomasses having activities towards not only cellulose, also hemicellulose and lignin. Isolates displaying multiple activities could potentially be applied on an industrial scale for the pretreatment or combined mechanical/chemical and biological treatment, of lignocellulosic biomass. Thus, the costs and environmental impacts associated with extensive pretreatment processes may be reduced by decreasing the amount of chemicals and or energy required. Here I propose the application of such lignocellulase producing bacteria during transportation and storage of lignocelluosic biomasses; or the potential application of such bacteria after shorter lignocellulosic biomass pretreatments such as mechanical treatments to lower pretreatment costs. There are a number of mechanical and chemical pretreatment routes and often combined mechanical and chemical routes which further increase pretreatment costs. Additionally, biological pretreatments have thus far included the application of white, brown and soft rot fungi, with potentially very limited costs;

however, the use of these strains has proved to be very time consuming due to slow growth rates and activities (Sun and Cheng, 2002; Tengerdy and Szakacs, 2003; Cardona and Sanchez, 2007). If we add bacteria, the process could be potentially shorter due to more rapid growth rates.

Moreover, there are few studies that have identified and characterized bacterial lignin-degrading enzymes and thus they have not been readily considered for the decomposition of lignins. The first lignin peroxidase gene was recently identified and characterized in 2011 by Ahmad *et al.*, in *Rhodococcus jostii* RHA1 (Ahmad *et al.*, 2011). The results suggest modification of lignin; this was shown by the increase in reflectance of pure lignin at 280 nm. Also, FTIR analysis, revealed preference for hemicellulose compared to lignin for the same isolates, however a change in lignin was evident when the area under the curves were compared to the cellulase positive control (*C. xylanilytica*). Barley straw was the biomass used for FTIR analysis and is shown in literature to contain approximately 14-15% mass as lignin, and 24-29% mass hemicellulose, while 31-34% cellulose (Husseien *et al.*, 2007). Although we cannot say lignin was degraded, I propose modification to allow for access to the hemicellulose and cellulose. I did however, attempt to measure lignin activities using microplate developed assays for lignin peroxidases (LiP), manganese peroxidases (MnP), laccases (Lac) and lipases (Bugg *et al.*, 2011); however, the results were inconclusive because of a lack of ability to individually optimize the strains for enzymatic expression. Thus, future work will be focused on the investigation of lignase genes with focus on one or two strains displaying lignin modification. Once again, parameters such as pH, temperature and atmosphere should be examined in the expression.

Recently, I have designed two degenerate primers (forward, D-FW 5"-GGNTTYGTNGAYGGNCANGARA-3" and reverse, D-REV 5"-HATYAAGTANTGNCCGTARTC-3), which are currently being used to „fish" for new uncharacterized peroxidases in those isolates displaying lignin modification. These degenerate primers were designed based on the homologous regions of peroxidase genes from a variety of bacterial peroxidases. Thus, if lignin peroxidase-like genes are

found, cloning and expression in an *E.coli* host systems will be done for biochemical characterization. If the search for lignase genes is unsuccessful, purification of enzyme extracts displaying lignin modification is required to further characterize the activities due to the variety of enzymes associated with lignin degradation and modification. With the identification and biochemical characterization of lignin-degrading enzymes there is potential for expression in other known efficient cellulose and hemicellulose degrading bacteria such as *Clostridium thermocellum* to enhance the efficiency and repertoire of biomass degrading enzymes.

The remaining focus of my studies was to utilize molecular biology techniques to further improve a known cellulase-producing bacterial system. I chose the well-known anaerobic, thermophilic *Clostridium thermocellum,* which is known for its high cellulase activity towards microcrystalline cellulose, comparable to that of *Trichoderma reesei* (Ng and Zeikus, 1981). Furthermore, there were three other main advantages for choosing this strain over *T. reesei*. *C. thermocellum* is ethanogenic, thus it can ferment 6-carbons to ethanol along with other by-products. Additionally its anaerobic and thermophilic nature also makes using this strain more cost effective during the industrial production of biofuels from lignocellulosic biomass. However, with this unique system there are several limitations holding back its industrial application which were discussed throughout this thesis. One such limitation is the metabolism of carbons by *C. thermocellum*; it can only ferment the 6-carbon sugars, and 5-carbon sugars found in hemicelluloses are not fermented.

Thus, my next project focused on the improvement of cellulose hydrolysis in *C. thermocellum,* by the relief of end-product inhibition. The addition of exogenous β-glucosidase from *Aspergillus niger* was shown to increase cellulase production (Lamed, *et al.*, 1990). Thus, I chose to express β-glucosidase in *C. thermocellum*. Due to the difficulty in genetic modification of *C. thermocellum* and due to time constraints I chose to first increase copy numbers of β-glucosidase A (*bgl*A) by cloning and expression in a shuttle vector (pIKM1). There would be no insertion to the genomic DNA due to a lack of recombinant

systems for thermophilic anaerobes. During this study, I was able to design a modified electroporation protocol to be used in future studies for *C. thermocellum* genetic modification. I also, expressed *bgl*A from the shuttle vector and saw a significant increase in β-glucosidase and total cellulase activities. However, during this study I note two important factors which lend knowledge towards our future studies.

Firstly, as can be expected for shuttle vectors, after 5 continuous generations without antibiotics there were no detectable transformants. The use of this transformed strain in industry would require addition of large amounts of antibiotics, such as the ampicillin used here. Thus, I propose future work includes the development of a recombinant system carrying β-glucosidase. In 2000, Mai and Weigel used PUC-based suicide plasmids, pUXK and pUXKK, to integrate and express a *C. thermocellum* cellobiohydrolase gene (*cbhA*) into the xylanase gene (*xyl*) of a *Thermoanaerobacterium spp.* (Mai and Weigel, 2000).

Secondly, an increase in total cellulase activity does not appear to have a direct effect on ethanol production, as was shown by our results in this study. However, I will further test the effects on ethanol through more time sensitive sampling of the ethanol in continuous fermentation. That is, fermentation which with the continuous replacement of substrates and nutrients and the removal of toxic or inhibitory end-products. Thus, one can compare with the ethanol produced before reaching toxic levels. Additionally, I can measure other valued endo-products such as hydrogen, as well as lactic and acetic acids to assess the ability of the transformant compared to the wild-type.

The variability seen in ethanol production between wild-type (WT) and transformed (+MC*bgl*A) *C. thermocellum* suggests a requirement for further engineering. Literature suggests ethanol and organic acid production is toxic for ethanogenic bacteria (Lynd, 1989; Rani and Seenayya, 1999; Burdette *et al.*, 2002; Zeng *et al.*, 2004). I propose that if a considerable increase in tolerance to ethanol and or organic acids is developed in *C. thermocellum* harbouring increased copies of β-glucosidase, I will also see an increase in ethanol titres. Studies have shown that increase in ethanol tolerance alone can increase ethanol

titres; however there is some controversy here (Shao *et al.*, 2011; Williams *et al.*, 2007). There is also an undefined tolerance to ethanol in *C. thermocellum*. Thus, I cannot say if the ethanol I saw produced is in fact at a toxic/inhibitory level.

Moreover, knockout systems such as the Clostron have been developed and future work to improve ethanol titre in addition to greater cellulase activities should include knocking out genes involved in acetate and lactate production, such as phosphotransacetylase (*pta*) and L-lactate dehydrogenase (*L-ldh*), respectively. Researchers have shown that the knockout of *pta* and *L-ldh* alone in *C. thermocellum* and *T. saccharolyticum*, respectively, allowed for increased tolerance to greater amounts of acetate (Tripathi, *et al.*, 2010) or lactate (Desai *et al.*, 2004), respectively. Thus, if future work can combine more resistant strains, produced via knockout or mutagenesis, with increased copy numbers of beta-glucosidase or cellobiophosphorylases a more industrially viable system could be applied for biofuel production and could be incorporated into co-cultures such as that developed in my last study.

Therefore, in my last study, I investigated the development of a stable co-culture of *C. thermocellum* and *T. saccharolyticum*, which could improve consolidated bioprocessing in the production of biofuels. Two strains working synergistically have the potential to complete multiple tasks such as in this case, the fermentation of both 5- and 6-carbon sugars and ultimately increase ethanol titres. Using PCR-based biomarkers I found that the stability depended on our development of a media with polysaccharide sugars such as xylan and Avicel, versus media containing reduced sugars.

This co-culture system developed here also lends knowledge for future improvements. Once again, I propose that the application of strains more resistant to toxic end-products such as ethanol and organic acid by-products could potentially prolong continuous co-culture generations. Thus, it is highly valuable to the anaerobic thermophilic research studies presented here if through mutation and selection or metabolic engineering strains with increased resilience to end-products be developed.

Additionally, my current and continuing future work related but not presented here has been involved in the collaborative development of a fused *C. thermocellum* and *T. saccharolyticum* strain; whereby protoplast formation allows the genetic recombination between their genomes. Fusions will be screened for the ability to ferment 5- and 6-carbon sugars. Also, the genetic stability of such a fused strain will be tested using my biomarker system, as was developed during the co-culture study. Such a fusion, could present more industrial potential if the ethanol titres are comparable to our co-culture system. In an industrial setting, the growth and maintenance of one strain could be simpler and thus, more cost effective.

Finally, with the success of my tansformation protocol one could potentially introduce unique genes such as lignase, xylanase or cellulase genes exploited from further studies on our isolates. *C. thermcellum* represents a unique host because of the cellulosome. If the type-I dockerin sequence is cloned and ligated to target genes it may be possible to have the genes expressed as part of the cellulosome. Cellulases have been cloned for production of mini-cellulosomes and expressed in hosts such as *Bacillus subtilis* (Cho *et al.*, 2004). Also, genetic modification in thermophilic anaerobic organisms continues to advance rendering greater opportunities for the development of economically feasible greener technologies to produce lignocellulosic biofuels.

In conclusion, all of the isolates, as well as the known cellulase-producing *C. thermocellum* transformant and co-culture must have their activities measured towards the use of reall hydrolysates such as corn husk or barely straw. This will allow one to determin the practibility of these bacteria in an industrial setting while addressing industrial environmental, technical and economic challenges.

References

Ahmad, M., Roberts, J.N., Hardiman, E.M., Singh, R., Eltis, L.D. and Bugg, T.D. (2011) Identification of DypB from Rhodococcus jostii RHA1 as a lignin peroxidase. *Biochem* **50**, 5096-5107.

Bugg, T.D.H., Ahmad, M., Hardiman, E.M. and Rahmanpour, R. 2011. Pathways for degradation of lignin in bacteria and fungi. *National Product Reports* **28,** 1871-1960.

Burdette, D.S., Jung, S. H., Shen, G.J., Hollingsworth, R.I. and Zeikus, J.G.. (2002) Physiological function of alcohol dehydrogenases and long-chain (C-30) fatty acids in alcohol tolerance of *Thermoanaerobacter ethanolicus*. *Appl Environ Microbiol* **68**, 1914–1918.

Cardona, C.A. and Sanchez, O.J. (2007). Fuel ethanol production: Process design trends and integration opportunities. *Bioresource Technol* **98**, 2415-2457.

Cho, H.Y., Yukawa, H., Inui, M., Doi, R.H. and Wong, S.L. (2004) Production of minicellulosomes from *Clostridium cellulovorans* in *Bacillus subtilis* WB800. *Appl Environ Microbiol* **70**, 5704-5707.

Fu, X., Liu, P., Lin, L., Hong, Y., Huang, X., Meng, X. and Liu, Z. (2010) A novel endoglucanase (Cel9P) from a marine bacterium Paenibacillus sp. BME-14. *Appl Biochem Biotechnol* **160**, 1627-1636.

George, J., Arunachalam, R., Paulkumar, K., Wesely, E.G., Shiburaj, S. and Annadurai, G. (2010) Characterization and phylogenetic analysis of cellulase producingStreptomyces noboritoensis SPKC1. *Interdiscip Sci* **2**, 205-212.

Hill, J., Nelson E., Tilman, D., Polasky, S., and Tiffany, D. (2006) Environmental,economic, and energetic costs and benefits of biodiesel and ethanol biofuels. *Proc Natl Acad Sci USA* 314,1598-1600.

Husseien, M., Amer, A.A., El-Maghraby, A. and Taha, N.A. (2007) Utilization of Barley Straw as a Source of a Activated Carbon for Removal of Methylene Blue from Aqueous Solution. *J Appl Sci Res* **3**, 1352-1358.

Lamed, R., Kenig, R., Morgenstern, E., Calzada, J.F., De Micheo, F., and Bayer, E.A. (1990) Efficient cellulose solubilisation by a combined cellulosome-β-glucosidase system. *Appl Biochem Biotechnol* **27**, 173-183.

Lynd, L.R. (1989) Production of ethanol from lignocellulosic materials using thermophilic bacteria. Critical evaluation of potential and review. *Adv Biochem Engin/Biotechnol* **38,** 1-52.

Lynd, L.R., van Zyl, W.H., McBride JE and Laser M. (2005) Consolidated bioprocessing of cellulosic biomass: an update. *Curr Opin Biotechnol* **16**, 577-583.

Mai, V. and Wiegel, J. (2000). Advances in development of a genetic system for Thermoanaerobacterium spp.: expression of genes encoding hydrolytic enzymes, development of a second shuttle vector, and integration of genes into the chromosome. *Appl Environ Microbiol* **66**, 4817–4821.

Ng, T.K. and Zeikus, J.G. (1981) Comparison of Extracellular Cellulase Activities of *Clostridium thermocellum* LQRI and *Trichoderma reesei* QM9414. *Appl Environ Microbiol* **42**, 231-240.

Okeke, B.C. and Lu, J. (2010) Characterization of a defined cellulolytic and xylanolytic bacterial consortium for bioprocessing of cellulose and hemicelluloses. *Appl Biochem Biotechnol* **163,** 869-881.

Pason, P., Kosugi, A., Waeonukul, R., Tachaapaikoon, C., Ratanakhanokchai, K., Arai, T., Murata, Y., Nakajima, J. and Mori, Y. (2010) Purification and characterization of a multienzyme complex produced by Paenibacillus curdlanolyticus B-6. *Appl Microbiol Biotechnol* **85**, 573-580.

Pastor, F.I., Pujol, X., Blanco, A., Vidal, T., Torres, A.L. and Diaz, P. (2001) Molecular cloning and characterization of a multidomain endoglucanase from *Paenibacillus* sp BP-23: evaluation of its performance in pulp refining. *Appl Microbiol Biotechnol* **55**, 61-68.

Philippidis, G.P., Smith, T.K., and Wyman, C.E. (1993) Study of the enzymatic hydrolysis of cellulose for production of fuel ethanol by the simultaneous saccharification and fermentation process. *Biotechnol Bioeng* **41**, 846-853.

Rani, K.S. and Seenayya, G. (1999) High ethanol tolerance of new isolates of *Clostridium thermocellum* strains SS21 and SS22. *World J Microbiol Biotechnol* **15**, 173–178.

Rivers, D.B. and Emert, G.H. (1988) Factors affecting the enzymatic hydrolysis of Municipal-solid-waste components. *Biotechnol Bioengin* **31**, 278-281.

Schneider, S.H. (1989) The Greenhouse Effect: Science and Policy. *Science* **243**, 771-781.

Shao, X., B. Raman, M. Zhu, J.R. Mielenz, S.D. Brown, A.M. Guss, and L. R. Lynd. 2011. Mutant selection and phenotypic and genetic characterization of ethanol-tolerant strains of *Clostridium thermocellum*. *Appl Microbiol Biotechnol* **92**, 641-652.

Sun, Y. and Cheng, J. (2002). Hydrolysis of lignocellulosic materials for ethanol production: A review. *Bioresource Technol* **83,** 1-11.

Tengerdy, R.P. and Szakacs, G. (2003). Bioconversion of lignocellulose in solid substrate fermentation. *Biochem Eng J* **13**, 169-179.

Waeonukul, R., Kyu, K.L., Sakka, K. and Ratanakhanokchai, K. (2009) Isolation and characterization of a multienzyme complex (cellulosome) of the Paenibacillus curdlanolyticus B-6 grown on Avicel under aerobic conditions. *J Biosci Bioeng* **107,** 610-614.

Williams, T.I., Combs, J.C., Lynn, B.C. and Strobel, H.J. (2007) Proteomic profile changes in membranes of ethanol-tolerant *Clostridium thermocellum*. *Appl Microbiol Biotechnol* **74**, 422–432.

Wyman, C.E., Dale, B.E., Elander, R.T., Hotzapple, M., Ladisch, M.R., and Lee, Y.Y. (2005) Coordinated development of leading biomass pretreatment technologies. *Bioresour Technol* **96,** 1959-1966.

Yang, D., Weng, H., Wang, M., Xu, W., Li, Y. and Yang, H. (2010) Cloning and expression of a novel thermostable cellulase from newly isolated *Bacillus subtilis* strain I15. *Mol Biol Rep* **37**, 1923-1929.

Zeng, A-P., Ross, A., Biebl, H., Tag, C., Günzel, B., Deckwer, W-D. (2004) Multiple product inhibition and growth modeling of *clostridium butyricum* and *klebsiella pneumoniae* in glycerol fermentation. *Biotechnol Bioengin* **44**, 902-911.

Publication List

Published:

Maki, M., Leung, K.T. and Qin, W. (2009) The prospects of cellulase-producing bacteria for the bioconversion of lignocellulosic biomass. *Int J Biol Sci* **5**, 500-516.

Dashtban, M.*, Maki, M.*, Leung, K.T., Mao, C., Qin, W. (2010) Cellulase activities in biomass conversion: measurement methods and comparison. *Crit Rev Biotechnol* **30**, 302-309.
(* represents equal contribution in first author writing)

Maki, M., Broere, M., Leung, K.T., Qin, W. (2011) Characterization of some efficient cellulase producing bacteria from paper mill sludges and organic fertilizers. *Int J Biochem Mol Biol* **2**, 146-154.
(**Co-author contribution**: Mike Broere assisted with the isolation and characterization of cellulase-producing bacteria)

Maki, M., Idrees, A., Leung, K.T., Qin, W. (2012) Newly isolated and characterized lignocellulase-producing bacteria with great application potential. *Int J Biochem Mol Biol* **22**, 156-166.
(**Co-author contribution:** Amna Idrees assisted in the isolation and characterization of lignocellulase-producing bacteria)

Maki, M., Armstrong, L., Leung, K.T., Qin, W. (2012) Increased expression of β-glucosidase A in *Clostridium thermocellum* 27405 significantly increases cellulase activity. *Bioengineered* (Accepted Aug. 23rd) (**Co-author contribution:** Lachlan Armstrong measured the ethanol production)

In preparation:

Maki, M., Armstrong, L., Leung, K.T., Qin, W. (2012) Development of a stable *Clostridium thermocellum* and *Thermoanaerobacterium saccharolyticum* co-culture for industrial production of bioethanol (**Co-author contribution:** Lachlan Armstrong measure the ethanol production)